EXPERIMENTAL CELL RESEARCH

Published under the auspices of

THE INTERNATIONAL SOCIETY FOR CELL BIOLOGY

UNIVERSITY OF HAWAII
LIBRARY
SEP 30 '55

EDITORS:

H. BAUER · T. CASPERSSON · A. ENGSTRÖM · E. FAURÉ-FREMIET

H. B. FELL · G. MONTALENTI · J. T. RANDALL · J. RUNNSTRÖM

F. O. SCHMITT · P. WEISS · R. W. G. WYCKOFF

VOLUME 9 · AUGUST 1955 · NUMBER 1

ACADEMIC PRESS INC., PUBLISHERS, NEW YORK, N.Y.

EXPERIMENTAL CELL RESEARCH

EDITORS:

H. Bauer, Max Planck Institut f. Meeresbiologie, Wilhelmshaven 23, Germany
T. Caspersson, Department of Cell Research, Karolinska Inst., Stockholm 60, Sweden
A. Engström, Department of Physical Cell Research, Karolinska Inst., Stockholm 60, Sweden
E. Fauré-Fremiet, Collège de France, Paris V:e, France
Honor B. Fell, Strangeways Laboratory, Cambridge, England
G. Montalenti, Istituto di Genetica dell' Università di Napoli, Naples, Italy
J. T. Randall, Wheatstone Physics Laboratory, King's College, London W. C. 2, England
J. Runnström, Wenner-Gren Institute for Experimental Biology, Stockholm Va., Sweden
F. O. Schmitt, Massachusetts Institute of Technology, Cambridge 39, Mass., U.S.A.
P. Weiss, Rockefeller Institute for Medical Research, New York 21, N.Y., U.S.A.
R. W. G. Wyckoff, Laboratory of Physical Biology, National Institute of Health, Bethesda, Md., U.S.A.

Editorial Secretaries: L. Ernster and Vera Reio, Norrtullsgatan 16, Stockholm Va., Sweden
Papers should be submitted to one of the editors or to the Editorial Office, Norrtullsgatan 16, Stockholm Va., Sweden.

ADVISORY BOARD:

W. Bloom (Chicago), J. Brachet (Bruxelles), F. Buchthal (Copenhagen), W. S. Bullough (London), A. Buzzati-Traverso (Pavia), R. Chambers (New York), E. Chargaff (New York), M. Chèvremont (Liège), J. F. Danielli (London), C. D. Darlington (London), J. N. Davidson (Glasgow), M. Demerec (Cold Spring Harbor, N.Y.), J. Engelbreth-Holm (Copenhagen), A. Fischer (Copenhagen), A. Forssberg (Stockholm), A. Frey-Wyssling (Zürich), N. Fries (Uppsala), S. Gard (Stockholm), R. J. Gautheret (Paris), L. Geitler (Wien), G. Gomori (Chicago), T. Gustafson (Stockholm), A. Haddow (London), E. Hammarsten (Stockholm), B. Hanström (Lund), E. Newton Harvey (Princeton, N. J.), L. V. Heilbrunn (Philadelphia, Pa), G. C. Heringa (Amsterdam), A. Hollaender (Oak Ridge, Tenn.), H. Holter (Copenhagen), J. Holtfreter (Rochester, N.Y.), T. Hultin (Stockholm), G. Klein (Stockholm), P. Koller (London), F. Lehmann (Bern), P. L'Héritier (Paris), G. Levi (Torino), P. E. Lindahl (Uppsala), O. Lindberg (Stockholm), G. Lindeberg (Uppsala), C. C. Lindegren (Carbondale, Ill.), R. J. Ludford (London), S. E. Luria (Urbana, Ill.), A. Lwoff (Paris), A. Mirsky (New York), H. Okkels (Copenhagen), O. Olivo (Bologna), A. W. Pollister (New York) G. Ranzi (Milano), W. J. Schmidt (Giessen), F. Schrader (New York), W. Seifriz (Philadelphia, Pa.), F. Sjöstrand (Stockholm), T. M. Sonneborn (Bloomington, Ind.), J. M. Sosa (Montevideo), J. Spek (Rostock), W. M. Stanley (Berkeley, Calif.), E. Suomalainen (Helsingfors), E. L. Tatum (Stanford University, Calif.), T. Teorell (Uppsala), A Tyler (Pasadena, Calif.), W. Wilbrandt (Bern), E. Zeuthen (Copenhagen).

Subscriptions

EXPERIMENTAL CELL RESEARCH

will be published in two volumes annually at $ 12: — per volume. Please send your subscription orders to Academic Press Inc., 125 East 23 St., New York 10, N.Y.

FERTILIZATION RATE REGULATORS

E. HULTIN and B. HAGSTRÖM

Institute for Organic Chemistry and Biochemistry and The Wenner-Gren Institute for Experimental Biology, University of Stockholm, Sweden

Received April 6, 1954

In a variety of organisms, such as algae, protozoa, sea urchins, polychaeta, molluscs and fishes, studies have been made of substances given off by the gametes which affect these cells during fertilization. Hartmann [6] introduced the term "gamones" for these substances, which have been studied especially in sea urchin material, among others by Hartmann and his group, Tyler, and by Runnström and his collaborators. Developments in this sphere have been treated in a number of review articles [15, 16, 17, 19].

The fertilization process is highly complicated, and the progress is dependent upon a number of factors which include the state of the eggs, the state of the spermatozoa, the concentration of the spermatozoa, and the presence of regulators. These various factors affect both the final percentage of fertilized eggs and the time which elapses between the insemination and the fusion of a spermatozoon with an egg.

Some of these influences have been investigated previously. Thus, the decrease in the final proportion of fertilized eggs caused by the ageing of the sperm has been studied by Lillie [12] and recently by Wicklund [23, 24], by Wicklund and Jonsson [26], and by Tyler [21].

The effect of the sperm concentration on the final percentage of fertilized eggs was observed by Lillie [12]. The influence of various regulators on the life period of the spermatozoa and on the final percentage of fertilized eggs has been the subject of several investigations (see, e.g., [10, 15, 18, 21, 23, 24, 25, 26] and the references given in these papers).

Thus, a great many investigations have been made on the influence of various factors on the final number of fertilized eggs. However, the fertilization rate has not been studied until recently. The pioneer work was done by Rothschild and Swann [14], who measured the fertilization rate at various sperm concentrations and calculated the average number of collisions between the spermatozoa and one egg before the egg was fertilized. Fertilization rates were also measured by Rothschild [13] with the aim of calculating the speed at which the block to polyspermy is formed.

It seems to be important that the investigations on the influence of various substances on the final percentage of fertilized eggs be supplemented by determining their effect on the fertilization rate. The present investigation constitutes a study of the substances given off by the sperm of the sea urchin and the effects of these substances on the rate of fertilization and on the final percentage of eggs fertilized.

As a prerequisite for this study, it was essential to be able to determine the relative number of eggs fertilized at different times after the insemination. A method for this purpose was first described by Rothshild and Swann [13, 14]. Another more convenient method has been worked out by Hagström and Hagström on the basis of previous experience [4], and it was used in this investigation with minor modifications.

Determination of the Proportion of Eggs Fertilized at Different Times

The ovaries from each animal was placed in separate dishes containing filtered sea-water. Samples were removed for microscopical examination, and females containing oocytes were rejected. After the eggs were shed, the ovaries were discarded and the eggs were washed by sedimentation.

The egg suspensions were so divided that as nearly as possible the same number of eggs was used in each experiment. A sperm suspension was prepared and kept in the cold (6–8°C) during the experiment. The sperm concentration was adjusted so that the final concentration at the insemination was 2.6×10^6/ml. This concentration was high in relation to the number of eggs.

A number of beakers were prepared containing sodium lauryl sulphate.[1] In a final concentration of 0.0007 per cent this substance immediately inhibited the movements of the spermatozoa. In several experiments, when the inseminated egg suspension was poured into a beaker with sodium lauryl sulphate solution 3 seconds after the insemination, about 95 per cent of the fertilizable eggs were still unfertilized. Sodium lauryl sulphate thus kills the spermatozoa rapidly enough for these measurements. The eggs were not injured by the dilute lauryl sulphate solution used.

The control egg suspension was inseminated; and after certain time intervals (3, 5, 10, 20 etc. seconds) 10 ml of the suspension was transferred to one of the beakers containing sodium lauryl sulphate. A part of the suspension was allowed to develop without addition of inhibitor. The other suspensions were treated in the same manner, except that, before the insemination, an addition was made of the substance, the effect of which on the fertilization rate was to be tested. In some experiments the regulator was allowed to act on the eggs for 5 minutes before the insemination, while in others the sperm was added immediately after the regulator. The cultures were permitted to develop for one to two hours, which faciliated the counting of fertilized and unfertilized eggs. About 250 eggs were counted in each culture.

[1] $C_{12}H_{25}OSO_3Na$, in the U.S.A. erroneously called sodium lauryl sulfonate; the sulphonate $C_{12}H_{25}SO_3Na$ is another chemical compound which is not yet commercially available.

In the comparative determinations of the effects of various regulators, the influence of the state of the sperm was eliminated by using the same sperm in all the experiments of a given series; the drain sperm[1] was kept undiluted and the experiments in a given series were commenced at such short intervals that deterioration of the drain sperm caused by ageing could have been of no significance. The time required for an experiment, including a control and 4–5 series with regulators, was about 20 minutes.

In experiments including rate determinations it is, of course, very important to carry out the experiments at a constant temperature. Unfortunately, we had no suitable thermostat available. However, in each of our comparative series there was no significant difference in the temperature of the individual fertilization mixtures. The temperature range for our different series was 14–16°C.

It has been surmised by Rothschild and Swann [14] that deterioration of the dilute sperm in the fertilization mixture may decrease the rate of fertilization, at least after some time. It will be emphasized in the following that the rate of fertilization depends not only on the state of the eggs but also on the state of the sperm. However, in comparative determinations of the effects of regulators, it is essential that the state of the sperm and the eggs is the same at the beginning of each individual experiment. Alterations in the sperm during the experiment will then either be the same in all experiments or reflect the influence of a regulator.

Calculation of the Fertilization Rate

If it be assumed[2] that in a fertilization experiment the egg material is uniform, so that each egg has the same probability of fertilization within a given time interval, then the following expression holds for the fertilization of a large number of eggs (cf. [14]):

$$\mathrm{d}n = -nk \cdot \mathrm{d}t, \tag{1}$$

where n = number of still unfertilized eggs; k = rate constant (the probability that one egg will be fertilized within unit time); t = time.

If the total number of eggs is n_0, we have by integration

$$n = n_0 \cdot \mathrm{e}^{-kt} \tag{2}$$

---

[1] The term *drain sperm* is suggested as a substitute for the erroneous common term *dry sperm*, which can only imply that the medium is removed or the sperm is desiccated.

[2] This is an assumption which enables a simple mathemathical treatment (cf. radioactivity). It must be emphasized that this assumption cannot be strictly valid for the reaction mechanism in fertilization, since an egg which has been in contact with sperm for a short time without being fertilized is not in exactly the same state as if it had not been in contact with sperm. The circumstances are evidently otherwise than in the radioactive decay, where the probabilities of disintegration are exactly equal for individual atoms of the same kind at any time. Another method for the mathematical treatment will soon be published by the present authors [8].

The fertilization rate may be expressed by the constant k, although it is more informative to employ the half fertilization period T, the time necessary for the fertilization of half the total number of eggs.

The relation between k and T is

$$kT = \ln 2. \tag{3}$$

Taking logarithms, we obtain from (2)

$$\log \frac{n}{n_0} = -(\log e)kt. \tag{4}$$

If, therefore, the logarithm of the fraction of unfertilized eggs $\frac{n}{n_0}$ is plotted against the time t, the experimental values should lie about a straight line, provided that our initial assumptions are satisfied.

It is frequently observed that all eggs are not fertilized. This indicates that the egg material is not uniform and that eggs in two different states are present. If it is desired to employ equations (1), (2) and (4) to express the course of fertilization in such a case, it is necessary to let n represent the number of fertilizable eggs (which, in a given experiment, may depend not only on the state of the eggs but also on that of the sperm and on effectors as will be shown later and as, indeed, has been known for some time).

If equation (4) is employed, and if the number of unfertilized eggs at time t is n_t and that after a long period n_∞, the equation takes the following form:

$$\log \frac{n_t - n_\infty}{n_0 - n_\infty} = -(\log e)\ kt. \tag{5}$$

This equation may be rewritten as:

$$\log (n_t/n_0 - n_\infty/n_0) = -(\log e)\ kt + \log (1 - n_\infty/n_0) \tag{6}$$

A plot of $\log (n_t/n_0 - n_\infty/n_0)$ versus t with experimental data is given in Fig. 1, where the points lie approximately on a straight line.

Nevertheless, in the measurements of fertilization rate carried out by the authors with sea urchin material on the west coast of Sweden in July and August 1953, the use of equation (6) generally gave plots of the type shown in Fig. 2: the points do not lie on a straight line, but on a curve of progressively decreasing slope. The obvious interpretation of this finding appears to be that the egg samples were very heterogeneous, containing all possible variants between rapidly fertilized and slowly

fertilized eggs. In other words, the probability that fertilization will take place within a given time interval is different for different eggs under identical experimental conditions.

It is, however, reasonable to suppose that cases may be encountered where the probabilities of fertilization tend to group themselves about a few definite values. That such cases actually occur is indicated by the experiments to be described later.

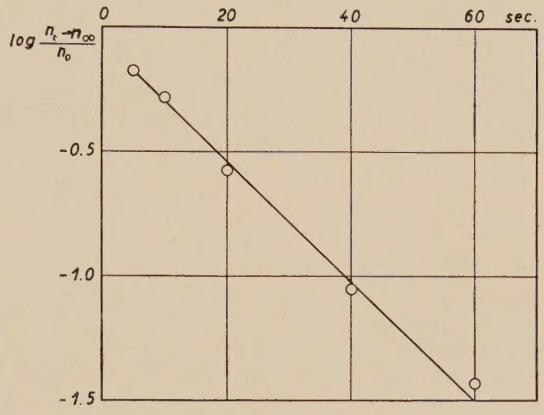


Fig. 1. All fertilizable eggs have the same half fertilization period.

The simplest interpretation of Fig. 2 is, therefore, that there are two varieties of eggs, one with a high fertilization rate, i.e. a high probability of fertilization within a given time interval, which implies a short half period, and one group with a low fertilization rate and hence a long half period. A more detailed analysis of Fig. 2, which will be described below, supports this interpretation.

The two groups of eggs with different fertilization rates need not be regarded as uniform in the strict mathematical sense with respect to their probabilities of fertilization in the manner of radioactive atoms with respect to degradation. On the contrary, it must be supposed that a certain statistical scatter about a mean is present, as with other biological data. The study of this statistical variation lies beyond the scope of the present work, although it may form the object of a future investigation [8].

If we do not initially assume the occurrence of more "types" of egg than we find essential, and if we also disregard the scatter about the mean values for the half period, which appear to be reasonable approximations in our first approach to the problem, the reasoning may be continued in the following manner.

The distribution of the eggs between the two categories "rapidly fertilizable" and "slowly fertilizable" may easily be calculated graphically [1, 3]. Commencing with the lower fertilization rate, we draw an asymptote as shown in Fig. 2. We thereby take into consideration that the accuracy is not the same for all points but decreases at the same time as the number of unfertilized eggs decreases. It would be advantageous to have more experimental points than are shown in the figure, e.g. also at 80 and 100 seconds.

The intercept of the asymptote and the ordinate axis ($t = 0$) gives the logarithm

of the fraction of slowly fertilizable eggs, the fertilization rate of which may be calculated from the slope.

It is easiest to calculate the half period, the time required for a diminution in the logarithm of the fraction of unfertilized eggs by $0.30 = \log 2$.

The fraction of eggs with the higher fertilization rate, n_r/n_0, is obtained by subtracting from the fraction of fertilizable eggs $(1 - n_\infty/n_0)$ that of slowly fertilizable eggs, n_s/n_0. The half period can be calculated as follows:

$$n_{s,t} = n_s \cdot e^{-\frac{\ln 2}{T}t} \tag{7}$$

and, if we take the relative number of eggs by dividing with n_0 and substitute 2 for e as basis for the exponential expression in order to simplify calculations on a slide-rule, we get, since $2 = e^{\ln 2}$

$$n_{s,t}/n_0 = \frac{n_s/n_0}{2^{t/T}} \cdot \tag{8}$$

For each time at which measurements have been made, equation (8) is used to calculate the fraction of remaining unfertilized eggs with long half period $n_{s,t}/n_0$. This calculation may readily be performed on a slide-rule with an e-log scale. The values so obtained are subtracted from the corresponding figures for unfertilized but fertilizable eggs, which gives the fractions of still unfertilized eggs of high fertilization rate, $n_{r,t}$. The logarithms of these fractions are plotted against the corresponding times and a straight line is fitted to the points as shown in Fig. 3. It is advisable to have more points than are shown in the figure, where an additional point at, say, 15 seconds would have been useful. The half period may be most conveniently read from the graph as the time necessary for a diminution of the logarithm of the fraction of unfertilized eggs with high fertilization rate by $0.30 = \log 2$.

When the line is extrapolated to $t = 0$, it is usually found that the intercept on the ordinate axis is not equal to $\log n_r/n_0$. If a line is drawn through $\log n_r/n_0$ parallel to the time axis, this cuts the experimental line at a point of abscissa t_0, which gives a time correction. The correction is composed partly of errors in the determination of the times at which the suspensions of sperm and eggs are mixed and at which the fertilization is broken off (experimental errors) and partly of the time required for the passage of the spermatozoa to and into the egg (physiological time lag). An example of the graphical computation of the time correction t_0 is given in Fig. 3. No advantage is gained, however, by introducing the corrected time $t–t_0$ in calculations made, for example, from equation (4).

If it is desired to check the graphical determination by successive approximations, the procedure is as follows. The intercept in Fig. 3 of the line through the experimental points with the ordinate axis $t = 0$ gives a fictitious fraction of readily fertilized eggs. (This may be greater than unity and serves only as an aid to calculation; its use is simpler than the application of the correction t_0 to all the time values.) With the aid of equation (8), a calculation is made for each time (uncorrected) of the fraction of rapidly fertilized eggs, which involves a substitution of n_r and $n_{r,t}$ for n_s and $n_{s,t}$ in the equation. The fraction of still unfertilized but fertilizable eggs at each time is then reduced by the calculated fraction of such eggs which are rapidly fertilized to give the fraction of still unfertilized eggs of low fertilization rate. The logarithms of

these values are plotted against the corresponding times and a straight line is fitted to the points as shown in Fig. 4. This process is repeated until two consecutive computations agree.

It has been remarked at an earlier stage that, whereas we speak of one class of readily fertilized and one of difficultly fertilized eggs, it would be unbiological to imagine that the eggs within each class had exactly the same probability of being

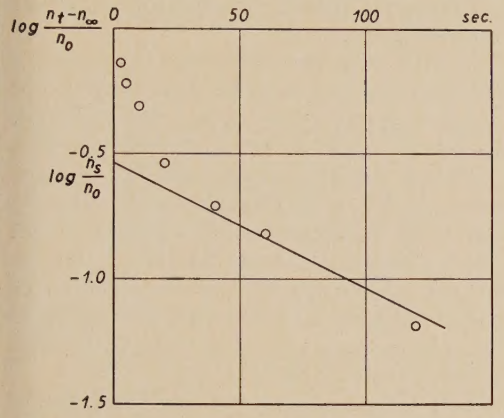
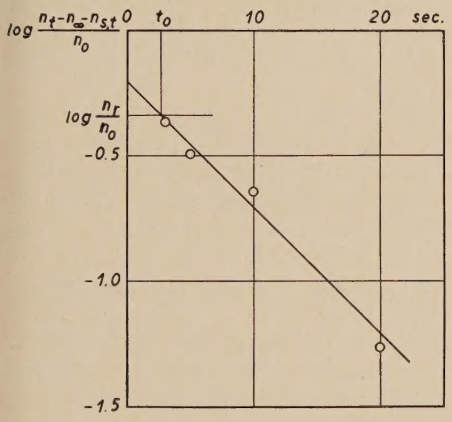


Fig. 2. The fertilizable eggs have not the same half fertilization period. At least two different groups are present.



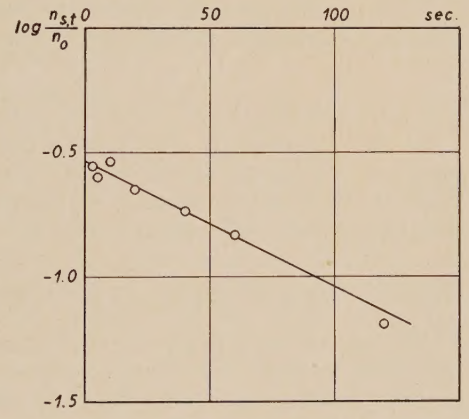


Fig. 3 (left). The category "rapidly fertilizable eggs". The slowly fertilizable eggs have been eliminated with the aid of the asymptote in Fig. 2.
Fig. 4 (right). The category "slowly fertilizable eggs". The rapidly fertilizable eggs have been eliminated with the aid of the fitted line in Fig. 3.

fertilized. It must be supposed, in fact, that the probabilities of fertilization of the individual eggs within a given time interval show a statistical variation.

In the treatment of our experimental data, which represented about 100 fertilization experiments made on some 20 occasions, we found it possible to assume the presence of a single category of eggs in only four cases. (These experiments were made with the same egg material, but with varying additions of several different

extracts: see Fig. 1.) In most cases it was necessary and sufficient to suppose that two categories were present. Only in two cases was there reason to assume the presence of three categories.

It should be mentioned at this point that, since in our experiments only about 250 eggs were counted at 7 or 8 times after the fertilization, the data give only a rough survey of the variations in the probability of fertilization. A more detailed picture could have been obtained only by taking samples at more frequent time intervals and preferably by counting more eggs on each occasion.

That such a clear-cut division of the eggs into only two categories was usually possible, may have been due to the fact that only the best animals were selected for the experiments, all females containing oocytes or cytolysed eggs being rejected.

In the following sections we describe experiments on the action of effectors. It is shown not only that the fertilization rate within a category of eggs may be altered, but also that some unfertilized eggs become capable of fertilization under the influence of the effectors, while some eggs with low fertilization rate acquire a high fertilization rate. This evidently implies that differences occur not only between, but also within the categories. As has been pointed out above, the classification of the eggs in different categories and the measurement of the half fertilization periods for these categories gives results which express collectively the properties of the eggs and the spermatozoa and the action of any effectors that may be present.

It should be mentioned here that, in the case of one category of eggs, Rothschild and Swann [14] give an instructive example of the statistical treatment of this problem according to the method of Fisher [2].

Sea Urchin Material

In the experiments described in this paper, only *Echinocardium cordatum* was used. Several experiments were also made with *Brissopsis lyrifera* and *Psammechinus miliaris*. These gave, however, no indication of species differences in the action of fertilization rate regulators.

Method for the Preparation from Sperm of Solutions Containing Regulators

The testicles were laid on a straining cloth in a funnel and the sperm was run into a measuring cylinder which was subsequently cooled. The sperm was then filtered through a Ulax filter (milk filter of cellulose). The spermatozoa were centrifuged down in a cold room with a centrifugal field of 1000 g. The supernatant, Sup. 1, was centrifuged again.

When it was desired to test the effect of high-molecular substances in the supernatant, this was dialysed overnight against a large volume of sea water. When low-molecular substances were under investigation, Sup. 1 was dialysed against four times its volume of sea water which was then used for the experiments. The liquid so obtained is called the "ultrafiltrate" in this paper, although its mode of preparation is not precisely ultrafiltration.

In some experiments the centrifuged sperm was suspended in about four volumes of sea water and centrifuged down after about 10 minutes. In this manner Sup. 2 was obtained, and so on.

Fertilization Rate Regulators are Given Off by the Spermatozoa to the Medium

On 9th July, 1953, 47 ml sperm, containing $75 \cdot 10^6$ spermatozoa/μl. was obtained from *Echinocardium cordatum*. After centrifugation, 3.7 g sperm sediment was obtained. This was suspended in 15 ml sea-water and centrifuged again after about 10 minutes. The supernatant liquid was tested for its content of fertilization rate regulators. One portion was dialysed overnight against a large volume of sea water, while

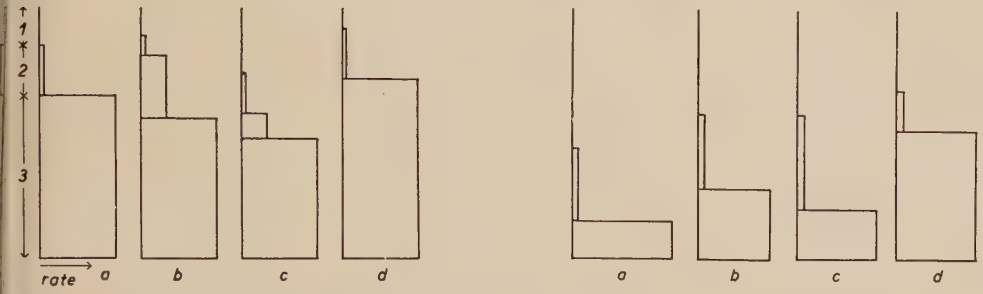


Fig. 5 (left). Release of fertilization rate regulators by the spermatozoa into the medium. *a*, control; *b*, with Sup. 2; *c*, with dialysed Sup. 2; *d*, with ultrafiltered Sup. 2. 1 = percentage of unfertilized eggs, 2 = percentage of slowly fertilizable eggs, 3 = percentage of rapidly fertilizable eggs. The width of each rectangle indicates the magnitude of the fertilization rate.
Fig. 6 (right). Release of fertilization rate effectors by the spermatozoa to the medium. *a*, control; *b*, with Sup. 2; *c*, with dialysed Sup. 2; *d*, with ultrafiltered Sup. 2.

another portion of 4 ml was dialysed against 25 ml sea water, the latter being retained as an "ultrafiltrate".

The original supernatant and the dialysed and ultrafiltered extracts were tested for their content of fertilization rate regulators. The volumes taken were 1,1 and 2 ml respectively for a total volume of 100 ml in the fertilization. The results are shown in Fig. 5, which gives the percentage distribution of the different categories of eggs and the respective fertilization rates. The heights of the rectangles, drawn one above the other, represent the percentages of the different categories of eggs, while the height of the vertical line above the rectangles indicates the percentage of unfertilized eggs. The widths of the rectangles represent the fertilization rates, which are inversely proportional to the half fertilization periods. The scale for the fertilization rate is so chosen that if the half period is one second, the width of the rectangle is the same as the total height of the rectangles plus that of the vertical line (100 per cent); if the half period is 10 seconds, the width is one-tenth of this height, and so on.

The results of a similar experiment carried out three days later are shown in Fig. 6.

The nitrogen content of the crude extract prepared in this manner (Sup. 2) was about 0.3 mg N/ml.

The experiments show that the spermatozoa release fertilization rate regulators to the surrounding medium. These regulators influence the fertilization even in great dilution. The figures indicate that the effect consists not only in a change in the proportion of fertilizable eggs, but also in an alteration of the ratio of rapidly fertilized to slowly fertilized eggs.

It has been shown previously [9] that sperm can give off nucleoproteins to their surrounding medium, and that nucleates inhibit or delay fertilization [5].

It might be tempting to conclude from an examination of Figs. 5 and 6 that Sup. 2 contains two fertilization regulators, a high-molecular, inhibiting factor and a low-molecular promoting factor. The results of subsequent experiments, however, show that such a conclusion would be premature (cf. Figs. 8 and 10).

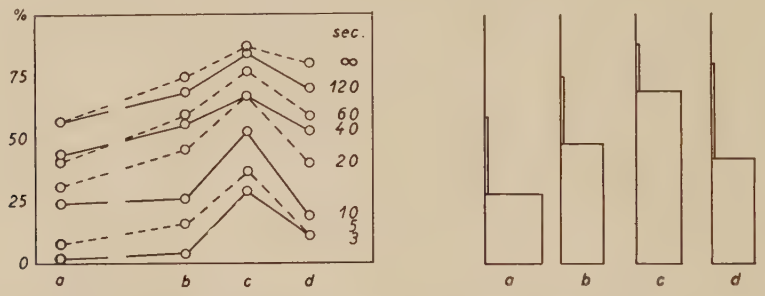


Fig. 7 (left). Percentage of fertilized eggs at different times in the presence of ultrafiltrate in different concentrations. *a*, control without ultrafiltrate; *b*, with ultrafiltrate 1:100; *c*, with ultrafiltrate 3:100; *d*, with ultrafiltrate 10:100.

Fig. 8 (right). Fertilization rate in the presence of ultrafiltrate in different concentrations. *a*, control without ultrafiltrate; *b*, with ultrafiltrate 1:100; *c*, with ultrafiltrate 3:100; *d*, with ultrafiltrate 10:100.

Activating and Inhibiting Effects of Ultrafiltrates from Sperm Medium

On 15th July, 1953, we obtained from 330 specimens of *Echinocardium cordatum*, 110 ml sperm. This, on centrifugation, yielded 14 g sperm sediment, corresponding to about $110 \cdot 10^6$ spermatozoa/ml in the original sample. The supernatant was dialysed against about four times its volume of sea water and the ultrafiltrate was kept at $-10°C$ in a freezing chest. It was used for fertilization experiments on 24th July, 1953 in dilutions of 1:100, 3:100 and 10:100. The results of the fertilization rate experiments are shown in Figs. 7 and 8.

On 29th July, 1953 a further fertilization experiment was carried out with the same ultrafiltrate in the dilutions 0.1:100 and 3:100. The results are given in Fig. 9.

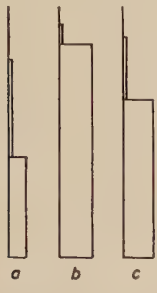
When eggs are treated with different dilutions of the ultrafiltrate, which evidently contains the low-molecular fraction of the substances in the sperm medium, a promotion is observed at lower concentrations and an inhibition at higher concentrations. With the biological material employed in the present experiments, the promotion consisted essentially in rendering some of the unfertilizable eggs fertilizable, while some of the slowly fertilizable eggs became rapidly fertilizable. The inhibition was manifested in the fact that the activation at higher concentrations was not as marked as at lower concentrations.

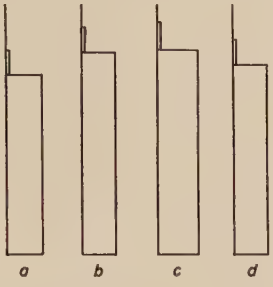
Two explanations are possible for these phenomena. They may be brought about by a single substance which promotes fertilization most effectively at a certain optimal concentration, or two substances in the ultrafiltrate may be concerned, one promoting and one inhibiting.

Activating and Inhibiting Effects in the Protein Fraction of Sperm Medium

A protein preparation from the sperm medium of *Echinocardium* cordatum was made in the following manner (the preparation was intended chiefly for the study of sperm cellulase):

From 582 animals was obtained 260 ml of sperm, which was filtered and centrifuged in the cold room. The sediment of spermatozoa weighed 30 g. The supernatant was poured off and the proteins were precipitated by the addition of 2 per cent tannic





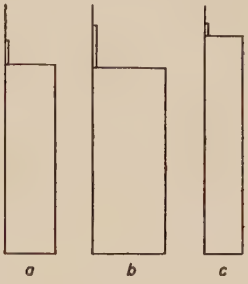


Fig. 9 (left). Fertilization rate in the presence of ultrafiltrate in different concentrations. *a*, control without ultrafiltrate; *b*, with ultrafiltrate 0.1:100; *c*, with ultrafiltrate 3:100.
Fig. 10 (center). Fertilization rate in the presence of a 0.12% solution of a protein preparation from the sperm medium in different concentrations. *a*, control without supplement; *b*, with protein solution 0.1:100; *c*, with protein solution 1:100; *d*, with protein solution 10:100.
Fig. 11 (right). Fertilization rate with washed sperm and in the presence of ultrafiltrate in different concentrations. *a*, control without ultrafiltrate; *b*, with ultrafiltrate 0.1:100; *c*, with ultrafiltrate 3:100.

acid until no further precipitation occurred. The precipitate was centrifuged down in the cold and the tannic acid was extracted with cold acetone according to the procedure of Weidenhagen [22]. The protein was collected on a Büchner funnel, washed with ether and dried *in vacuo*. The yield was 0.146 g. In order to determine the solubility of the preparation, 0.0247 g was extracted with 1.292 g water and the extract was centrifuged. The nitrogen content of the solution was 0.41 mg N/ml. The content of soluble cellulase in the preparation, which was determined for another purpose, was 15 mμ A/g [7, 11].

On 31st July, 1953, fertilization experiments were made, using a stock solution of the protein preparation of 19.6 mg in 15 ml sea water. Dilutions of 0.1:100, 1:100 and 10:100 were employed. The results are shown in Fig. 10.

Mention should be made at this point of an experiment performed on 15th July 1953, when the centrifuged sperm medium (Sup. 1) of *Echinocardium cordatum* was used without previous removal of the low-molecular fraction. Three fertilization experiments were made: a control, and experiments with eggs diluted with Sup. 1 in the dilutions 1:100 and 10:100. The percentages of fertilized eggs were 86, 90 and 77 respectively. At the same time a dialysed Sup. 2 (sperm spun down, suspended in 4 times their weight of sea-water and spun down again) was tested in a dilution of 0.5:100. This preparation, in which the high-molecular fraction was presumably

present in a higher dilution than in the other two solutions, gave the best fertilization, 95 per cent of fertilized eggs.

We may recall in this connection the earlier experiments of which the results are shown in Figs. 5 and 6. Here use was made of a dialysed solution (Sup. 2) of the relatively high-molecular substances given off by living spermatozoa to sea water in which they have been suspended after the first centrifugation.

The experiments show that the fraction of the sperm medium precipitable with tannic acid, which can reasonably be assumed to consist essentially of proteins, can promote fertilization in very high dilution. A smaller dilution, however, has an inhibitory action so strong that not only is the promoting effect neutralized, but less effective fertilization is obtained then in the control.

Concerning the high-molecular fraction also, two hypotheses may be entertained. Either there is a single substance which displays its greatest activity at a certain concentration and smaller activity at lower and higher concentrations, or there are two substances, one of which (possibly an enzyme) has a promoting effect, while the other is an inhibitor.

Fertilization with Washed Spermatozoa

The experiments described up to the present have shown that fertilization rate regulators are present in the sperm medium and can act in considerable dilution. The effect of washing on the fertilizing properties of the spermatozoa is therefore an obvious matter for investigation. Only a few experiments were carried out.

The same ultrafiltrate was employed as in the experiments described in the previous section.

On 29th July, 1953 a series of six experiments was made, three with washed and three with unwashed sperm. The washing procedure consisted in diluting the sperm with 5 volumes of sea water, centrifuging in a hand-driven centrifuge for 3 minutes, removing the supernatant and suspending the spermatozoa in sea water. With both washed and unwashed sperm, experiments were made without additions and in the presence of ultrafiltrate diluted 0.1:100 and 3:100. The results of the three experiments with unwashed material are shown in Fig. 9, and the other results in Fig. 11.

The results appear at first sight somewhat difficult to explain. In view, however, of the findings already described, a reasonable interpretation appears to be the following:

In the experiments with unwashed sperm, such large quantities of regulators have been added together with the sperm that a concentration of ultrafiltrate considerably lower than 3:100 gives the optimal quantity of rapidly fertilized eggs (the optimal concentration is, of course, probably not identical with the lower concentration (0.1:100 used in the experiment).

In the fertilization, the volume of sperm corresponded to about 3 ml ultrafiltrate when allowance is made for the dilution in the preparation of the latter. The amount of regulators added with the sperm was not, however, equivalent to that in 3 ml ultrafiltrate, since any high-molecular effectors present were removed in the ultrafiltration.

In the experiments with washed sperm, where appreciably smaller quantities of

effectors were added with the sperm, a higher concentration of ultrafiltrate was required than in the experiments with unwashed sperm to give the highest proportion of rapidly fertilized eggs: this optimal concentration was considerably greater than 0.1:100. A further experiment with washed sperm and an ultrafiltrate concentration greater than 3:100 would have been desirable; the results described in earlier sections lead us to suspect that an inhibition would have been observed in such an experiment, at least in relation to the quantities of rapidly fertilized and total fertilized eggs at the concentration 3:100.

An examination of the fertilization rates shows that, for both unwashed and washed sperm, the concentration for optimal fertilization rate with the rapidly fertilized eggs lay below 3:100, since in both cases the dilution 0.1:100 gave a higher fertilization rate than the dilution 3:100 and than the control. This is seen most clearly in the experiment with washed sperm.

While no far-reaching conclusions may be drawn from this series of experiments, we may venture to suggest that a single substance need not be responsible for the two effects, increases in the percentages of rapidly fertilized and fertilizable eggs on the one hand, and an increase in the fertilization rate for the rapidly fertilized eggs on the other.

Effect of Ultrafiltered Sperm Medium on the Durability of the Sperm

In two parallel series of experiments, sperm of *Echinocardium cordatum* was diluted with sea-water and with a diluted (3:100) ultrafiltrate of sperm medium (Sup. 1) prepared in the manner previously described. After different times, sperm samples were removed for fertilization experiments. The results are shown in Fig. 12.

It is well known from earlier investigations that the sperm medium and proteins have the power of delaying the ageing of the sperm [10, 20, 21, 23, 24, 26]. From the figure it may be concluded that ultrafiltered sperm medium also contains a substance which acts similarly in considerable dilution.

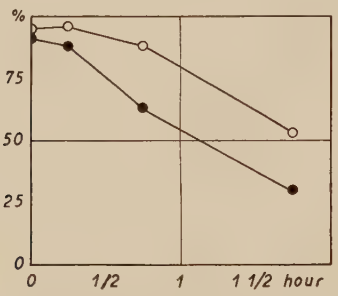
An Ether-soluble Fertilization-promoting Substance in Ultrafiltered Sperm Medium

From 233 *Echinocardium cordatum*, we obtained 100 ml sperm. This was filtered and centrifuged. The sediment of spermatozoa weighed 10 g. The medium was dialysed against four times its volume of sea-water.

(a) 50 ml of ultrafiltrate was acidified and extracted three times with a mixture of 5 ml ether and 5 ml petroleum ether. The extract was transferred to sea-water in the following manner. After the solution had been dried with anhydrous sodium sulphate, the solvent was distilled off on a water-bath. The residue was extracted with ether and the extract was transferred to a test tube. The ether (2 ml) was evaporated almost completely and about 2 ml ethyl alcohol was added. The solution was evaporated almost to dryness and 3 ml sea-water was added. The solution was used in a dilution of 2:100.

(b) 50 ml of ultrafiltrate was made alkaline and extracted as in (a). The extract was transferred to sea-water in the same manner. The solution was employed in a dilution of 1:100.

(c) After the residue from the evaporation of the ether-petroleum ether extract in (b) had been extracted with ether, an extraction was carried out with alcohol. This extract (2 ml) was evaporated almost to dryness in a test tube and 3 ml sea water was added. The solution was used in a dilution of 2:100.



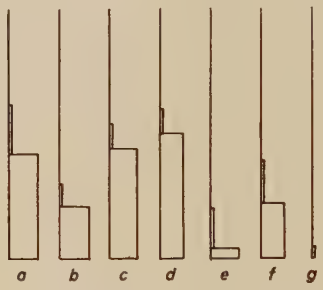


Fig. 12 (left). Ageing of sperm after dilution with (*a*) sea-water (dots), (*b*) ultrafiltrate (rings).
Fig. 13 (right). Fertilization rate in the presence of an ether-petroleum ether extract of ultrafiltered sperm medium. *a*, acid extraction; *b*, alkaline extraction with ether-petroleum ether; *c*, alkaline extraction with alcohol of residue from *b*, diluted 2:100; *d*, same as *c* but diluted 0.2:100; *e*, control with sea-water extract; *f*, with ultrafiltrate in dilution 1:100; *g*, control without supplement.

(d) The same solution as in (c) was used in the dilution 0.2:100.

(e) As a control, an extract of 50 ml sea water was made in the same manner as in (a). The solution was used in a dilution of 2:100.

(f) Fertilization experiments were also made with ultrafiltrate in the dilution 3:100.

(g) Fertilization experiments were made without additions.

The results are shown in Fig. 13.

It is apparent that substances soluble in lipid-solvents can exert a strong promoting effect upon the fertilization. In order to ensure that the fertilization process was not disturbed by the ether used in the transfer of the lipid substances to sea water, this was removed almost completely by distillation after the addition of alcohol. It is conceivable that the ether and petroleum ether used in the extraction of the ultrafiltrate, contained some substance which affected the fertilization even in great dilution. Such a substance would, however, presumably have been removed during the distillation of the solvent mixture. Since in experiment (e) an addition of "sea water extract" had some promoting effect on the fertilization, this must be regarded as the true control experiment with which the others should be compared.

All experiments with extracts of ultrafiltrate gave improved fertilization. It is conceivable that excessive concentrations were used in the fertilization experiments and that the fertilization-promoting substance at such high concentrations has a weaker promoting effect than at an optimal concentration. This is suggested by the following observations. The ether extract obtained in the alkaline extraction probably

contained the greater part of the ether-soluble substances and gave the smallest promoting effect. The subsequent extraction with alcohol presumably gave a weaker solution and the second such extraction gave the solution with the greatest promoting action.

Little can be said at present regarding the chemical nature of the ether-soluble substances. It was observed that the flasks used for the evaporations became coated with a fatty layer when the organic solvent was removed. Substances other than fatty acids must have been concerned, however, since the active material could be extracted from alkaline solution. It appears more reasonable to suppose that the substance is a phosphatide or a steroid.

SUMMARY

1. The investigation was carried out with *Echinocardium cordatum* on the west coast of Sweden in July and August, 1953.

2. The fertilizing power of spermatozoa is removed immediately in a 0.0007 per cent solution of sodium lauryl sulphate. This makes it possible in fertilization experiments to measure decreases in the proportion of unfertilized eggs during the period after insemination.

3. The proportion of fertilizable eggs is dependent upon the properties of the eggs and the spermatozoa and on any regulators that may be present.

4. The fertilization rate, the probability that a given egg will be fertilized within unit time, depends on the properties of the egg and the spermatozoa and may be influenced appreciably by fertilization rate regulators.

5. The individual fertilizable eggs in a group of animals in the same catch may be uniform with respect to fertilization rate, with due allowance for the statistical scatter usually found with biological material. Usually, however, the fertilization rates are highly varied.

6. In measurements of the relative (or percentage) distribution of eggs of different fertilization rates, the eggs could usually be assigned to three groups: unfertilized eggs, eggs of low fertilization rate and eggs of high fertilization rate.

4. Under the influence of fertilization rate regulators the proportions in which these three groups occur may be altered and the fertilization rates within the groups may be changed.

8. The spermatozoa release fertilization rate regulators into the medium, which act in considerable dilution.

9. Ultrafiltered sperm medium promotes fertilization in low concentrations by increasing the proportions of fertilizable eggs and rapidly fertilized eggs and by increasing the fertilization rate of rapidly fertilized eggs. If a certain optimal concentration of ultrafiltrate is exceeded, inhibitory effects appear.

10. The fraction of the sperm medium precipitable with tannic acid promotes fertilization in low concentrations. If, however, an optimal concentration is exceeded, an inhibitory action becomes evident.

11. In the study of fertilization rate regulators from sperm, account must be taken of the amounts of regulators accompanying the spermatozoa in their medium. It may be advantageous to employ washed spermatozoa.

12. Ultrafiltered sperm medium in considerable dilution can retard the ageing of the sperm.

13. Ultrafiltered sperm medium contains a low-molecular fertilizatiom promoting substance which is soluble in a mixture of ether and petroleum-ether.

The fertilization experiments were performed by one of the authors (B. H.), while the biochemical work and the mathematical treatment of the experimental results were carried out by the other (E. H.). The investigation was made at the Zoological Station of the Academy of Sciences at Kristineberg. We wish to express our thanks to Professor John Runnström for his kind interest in our work, to Dr. Gunnar Gustafson of the Zoological Station for providing the material and to Mrs. Britt Hagström for her assistance in the fertilization experiments.

REFERENCES

1. Daniels, F., Mathews, J. H., and Williams, J. W., Experimental Physical Chemistry, 4th ed., p. 329–330, 1949.
2. Fisher, R. A., *Trans. Roy. Soc. London* **A 222**, 309 (1922).
3. Flexner, L. B., Cowie, D. B., and Vosburgh, G. J., *Cold Spring Harbour Symposia Quant. Biol.* **13**, 88 (1948).
4. Hagström, B. and Hagström, B., *Exptl. Cell. Research* **6**, 479 (1954).
5. —— *Arkiv Zool.* [2] **7**, 579 (1955).
6. Hartmann, M. and Schartau, O., *Biol. Zentr.* **59**, 571 (1939).
7. Hultin, E., *Acta Chem. Scand.* **3**, 697 (1949).
8. Hultin, E. and Hagström, B. (To be published.)
9. Hultin, T., *Arkiv Kemi* **1**, 419 (1949).
10. Hyashi, T., *Biol. Bull.* **89**, 162 (1945).
11. Kristiansson, I., *Svensk Kem. Tidskr.* **62**, 133 (1950).
12. Lillie, F. R., *Proc. Natl. Acad. Sci. Wash.* **1**, 156 (1915).
13. Rothschild, Lord, *J. Exptl. Biol.* **30**, 57 (1953).
14. Rothschild, Lord and Swann, M. M., *ibid.* **28**, 403 (1951).
15. Runnström, J., *Advances in Enzymol.* **9**, 241 (1949).
16. —— *Harvey Lectures Ser.* **46**, 116 (1951).
17. —— *Symposia Soc. Exp. Biol.* **6**, 39 (1952).
18. Runnström, J., Monné, L. and Wicklund, E., *J. Colloid. Sci.* **1**, 421 (1946).
19. Tyler, A., *Physiol. Revs.* **28**, 180 (1948).
20. —— *Biol. Bull.* **99**, 324 (1950).
21. —— *ibid.* **104**, 224 (1953).
22. Weidenhagen, R., *Z. Verhandl. deutsch. Zucker-Ind.* **83**, 505 (1933).
23. Wicklund, E., *Arkiv Zool.* **42** A, No. 11 (1949).
24. —— *ibid.* (2), **3**, 333 (1952).
25. Wicklund, E. and Gustafson, T., *ibid.* **42** A, No. 12 (1949).
26. Wicklund, E. and Jonsson, I., *ibid.* (2), **2**, 559 (1951).

THE RELATION OF NUCLEIC ACID AND PROTEIN TO PANCREATIC SECRETION IN THE *RANA CLAMITANS* TADPOLE[1]

J. J. FREED[2, 3]

Department of Zoology, Columbia University, New York, U.S.A.

Received September 8, 1954

It has been shown by Caspersson and his coworkers [7, 8, 22] as well as by others [4, 5], that growing cells of a wide variety of types are characterized by the presence of large amounts of cytoplasmic nucleotides. Such an accumulation of nucleic acid, and the attendant cytological configuration, is considered to represent the system for cytoplasmic protein synthesis. Cells, such as gland cells, which secrete large quantities of protein but which undergo no permanent increase in cell mass, have been shown by a variety of techniques to have high contents of cytoplasmic pentose nucleic acid (PNA) [4, 7, 9, 11, 28]. These observations thus accord with those on growing cells in implicating PNA in the process of protein synthesis, rather than the more general phenomenon of growth.

The vertebrate pancreas would thus seem to be a favorable system for an attack on the problem of protein synthesis at the cytochemical level. Van Weel and Engel [32] were able to demonstrate that in the mammalian pancreas the content of cytologically visible zymogen granules was restored, after pilocarpine stimulation, at the same rate as enzyme synthesis proceeded. Hirsh [17], and Duthie [14] have shown, by microscopic observations *in vivo*, that the secretory process in the pancreas cell involves first a rapid extrusion of the granule material into the lumen of the gland, followed by a gradual increase in the number of the granules until a high content is again restored. This behavior of the cells has been confirmed by other workers, using fixed material [30, 32]. While the amphibian pancreas does not appear to be susceptible to pilocarpine stimulation [3], it has been reported to behave similarly in response to the stimulus of feeding [24].

[1] From a dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, in the Faculty of Pure Science of Columbia University.

[2] McCallum Foundation Fellow of Columbia University.

[3] Present address: The Institute for Cancer Research, and the Lankenau Hospital Research Institute, Philadelphia, Pa., U.S.A.

The ergastoplasm, the basophilic material characteristic of serous gland cells, has long been considered to be connected with the synthetic process, and variations in the basic staining of this material were reported to be connected with the secretory activity [2, 3, 30]. Since the development of modern cytochemical techniques, the PNA content of the ergastoplasmic material has been established both by ultraviolet absorption characteristics [9], and by the basic staining-ribonuclease method [4]. Caspersson and his associates [9] concluded that an increase in nucleotide absorption was associated with the secretory activity elicited by pilocarpine, while Noback and Montagna [25] using the basic staining-ribonuclease technique evaluated visually, concluded that both the extent and the intensity of the basophilic reaction were augmented during the restitution stage of the secretory cycle.

However, recent biochemical studies of the nucleic acid content of the pancreas after pilocarpine stimulation [11, 28], fail to show any variation in the ratio of PNA to DNA. On the assumption that the DNA content per nucleus is constant, this indicates no change in the amount of PNA per cell. Further, the isotopic studies of Hokin [18], and of Deken-Grenson [13] indicate that no turnover of PNA-phosphorus is associated with the synthesis of digestive enzymes.

The present study was designed to obtain information on the quantitative behavior of cytoplasmic nucleic acids and proteins, using cytophotometric methods combined with quantitative morphological analysis of the volumes of cell structures. Objective measurement of dye binding or absorption per cell was facilitated by the use of the tadpole pancreas, with its large cell size, homogeneous basophilia, and synchronous acinar behavior. In addition, the use of frozen-dried sections permitted the qualitative study of nucleic acid and protein distribution by means of cytochemical reactions to be made on material of high cytological quality.

EXPERIMENTAL

Material.—Second year tadpoles of *Rana clamitans* Latreille used in the experiments reported here were obtained from a single pond in the vicinity of Tenafly, New Jersey. The animals were maintained at 16°C in bowls of spring water.

Feeding experiments.—Animals were fasted for 21 days in frequent changes of spring water, subsequent feeding taking place in spring water containing approximately one cubic centimeter finely divided boiled egg yolk per liter. Alternatively, animals fasted for twenty-four hours were fed powdered agar while in spring water containing 0.1 per cent peptone broth powder. Controls were kept in clean spring water.

Preparation of sections.—The ventral lobe of the pancreas was quenched and frozen-dried at —40°C by the techniques described elsewhere [16]. After embedding in paraffin, ribbons of sections were cut at 16°C, at constant cutting speed to ensure uniformity of section thickness. Sections from the same ribbon were found to give mean cytoplasmic extinctions that agreed within the error of the photometric method. Sections were mounted on glass or quartz slides by the diacetin technique. After removal of paraffin by xylol or chloroform, ethanol (95 per cent) at 60°C for two hours was used to effect post-fixation, and to remove non-nucleoprotein substances.

Cytophotometric methods.—Measurements of absorption were made using instruments of the type described by Swift [31], or by Pollister [27]. The general procedure in making such absorption measurements has often been described, e.g. by Pollister [27]. In the visible portion of the spectrum, an objective numerical aperture of 1.25 was used, while the aperture of the condenser was held to approximately 0.3. Preparations were measured in mounting oil n_D 1.540 (R. P. Cargille Company) at which value scatter in the darkfield microscope was minimal. The method for obtaining a population of absorption measurements was held closely comparable to that used in the Chalkley method of volume estimation, making use of the same criteria. Slight adjustments of the stage were made to ensure that the area used for the measurement contained only non-granule cytoplasm. The measured areas were 3 to 4 μ in diameter. Twenty to 30 measurements were required to give a reproducible mean.

Naphthol yellow S, an acid dye, was used for the estimation of basic protein groups, according to the technique of Deitch [12]. Measurements were made at the 435 mμ mercury line. Azure B was used to measure nucleic acid phosphoric groups, according to Flax and Himes (15). Sections were stained two hours at 45°C in 0.25 mg/ml azure B bromide (Nat. Anil. Div., Cert. No. NA-214,92 per cent dye content), buffered at pH 4.0 with 1 per cent potassium acid phthalate. Extinction at 546 mμ was used as the measure of relative concentration, three point curves being used routinely to detect possible metachromatic shifts. When sections were previously treated with hot trichloroacetic acid (5 per cent at 90°C for 15 minutes), or ribonuclease (20), no stain was detectable in non-granule cytoplasm.

Ultraviolet absorption was measured using the 254 mμ mercury line, and N.A. 0.56 reflecting optics (American Optical Co.), with a refracting quartz ocular. Absorption was measured using preparations mounted in glycerin. The glycerin was then removed by absolute ethanol, the sections covered with a celloidin film to prevent loss, and allowed to dry. Five per cent trichloroacetic acid was used for 90 minutes at 60°C to extract nucleic acids, no further loss of absorbing material occurring after this time. After removal of the celloidin with alcohol-ether (1:1), the slides were rinsed in absolute ethanol, remounted in glycerin, and measured again. In most cases the non-specific light loss so obtained was from 30 to 40 per cent of the total, but in about one-fourth of the cases, values above 50 per cent were obtained. Thus it was surmised that extraction had produced changes in the protein structure. Darkfield observation of the preparations confirmed that notable differences in scattering power occurred from slide to slide, while a number of unextracted preparations were fairly uniform in their scattering. Therefore, the results reported in this study in the ultraviolet are for total absorption, and are used to

detect possible major changes in relative nucleic acid concentration not detected by corresponding changes in basophilia.

Quantitative morphological analysis.—Volumes of cell constituents were determined by the Chalkley method [10]. This method is based on the observation that if a pattern of points repeatedly be superimposed in a random manner on the image of a histological section, the ratio of the frequencies of "hits" on different structures will approach the ratio of their volumes as the number of counts increases. The use of a large array of pointers increases the speed of the method, and the use of a periodic arrangement does not appear to introduce error [16]. Counts were made of hits on the following: the nucleus, including the nucleolus; the nucleolus, including its associated chromatin; secretory granules; and non-granule cytoplasm, the last defined as the acinar cell material not assignable to the first three structures, and corresponding to the hyaline cytoplasm, including the mitochondria. Six hundred hits were considered a unit observation, and ten such observations per pancreas were made. A field was accepted or rejected for counting on the basis of the following criteria: (i) peripheral position, not under a blood layer during quenching, (ii) homogeneous cytoplasm, fairly homogeneous nucleoplasm, (iii) presence of unswollen, well preserved mitochondria. By passing completely around the periphery of the section a representative sample could be obtained. A number of sections were used for the determination on each organ. Nuclei were measured in absolute terms by means of a filar micrometer, and since no significant change in volume was detected from pancreas to pancreas, all values have been reported based on a standard nuclear volume of 200 μ^3, the mean for all nuclei measured. The standard error of the volume was calculated as follows:

$$\text{S.E.}_{\text{vol.}} \frac{\bar{X}}{\bar{N}} \sqrt{\left(\frac{\text{S.E.}}{\bar{X}}\, x\right)^2 + \left(\frac{\text{S.E.}}{\bar{N}}\, n\right)^2} \cdot 200\ \mu^3$$

where $\bar{X}$ is the mean frequency of hits on a given constituent, and $\bar{N}$ is the mean frequency of hits on nuclei; while the standard error of the frequency is that of the ten unit observations.

RESULTS

Qualitative observations.—The study of the chemical cytology of the pancreas cell was aided considerably by the use of successive treatments of the same sections by different cytochemical methods. The quality of fixation of the material was judged by reference to similar material prepared by fixation in osmium tetroxide, and sectioned in methacrylate [26].

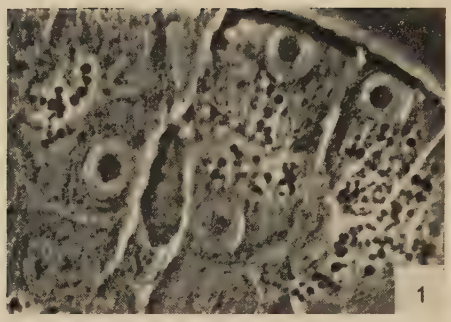
The ellipsoidal acinar cell nuclei contain DNA-protein concentrations of three varieties: (a) the nucleolus associated chromatin, (b) clumps of chromatin in the ground substance, and (c) a peripheral shell, applied to the nuclear envelope. This appearance (Fig. 6) is characteristic of Feulgen stained frozen-dried material. However, when the differential basic staining method of Flax and Himes [15] is used, the nucleolus associated ma-

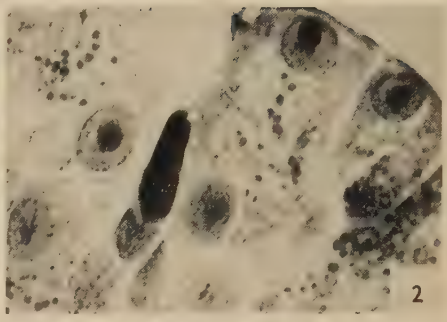
terial stains metachromatically (violet), while other clumps of similar size in the nucleus, but free of the nucleolus, are stained orthochromatically (blue). After ribonuclease digestion, the nucleolus associated material also stains orthochromatically. These observations suggest the presence of a different PNA/DNA ratio in these two regions, the relative amount of PNA being greater in the nucleolus-associated material. Under the conditions of quenching used, it is unlikely that this is a diffusion artefact, caused by loss of material from the nucleolus. The nucleolus itself is ovoid, and has a high phase density (Fig. 1), and strong nucleic acid-specific basophilia (Fig. 4). The intense staining with acid dye (and with the Millon reaction) indicates a high concentration of protein (Fig. 2).

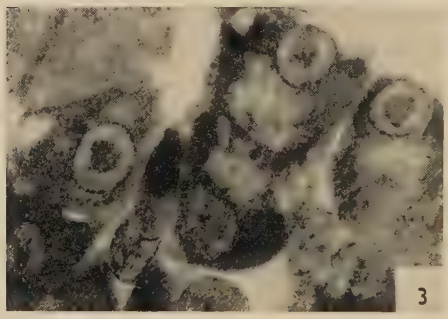
The mitochondria are threadlike, attaining a length of about five microns, and a width of up to two microns, and are denser than the surrounding cytoplasm when examined by phase contrast. (See Figs. 1 through 6.) They also take up more acid dye than the surrounding cytoplasm. While it is not possible to estimate the amount of PNA in such structures, they are certainly not regions of high nucleotide concentration, since in photomicrographs at 254 mμ they appear as lightly absorbing regions. After ribonuclease digestion, the pattern of relative absorption at 254 mμ is reversed, the mitochondria now absorbing more heavily. Upon fasting of the animals, changes occur, the mitochondria then appearing as short, thickened rods, frequently arranged in parallel bundles (Fig. 7). A few hours after the beginning of refeeding, as is the case in normal animals, the mitochondria are found to be threadlike, occurring in all parts of the cell (Fig. 8).

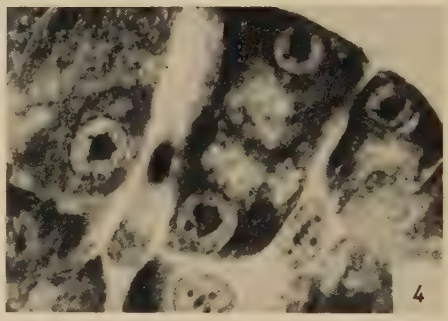
The secretory granules are spherical bodies of high refractive index, accumulated at the apical portions of the acinar cells, with diameters ranging from two microns down to the limit of resolution. They appear to be structures of high protein concentration, with no detectable nucleic acid (Figs. 1 through 6). Thus, they stain intensely with acid dyes and with the Millon reaction, while remaining unstained with basic dyes, and having no loss in absorption at 254 mμ after ribonuclease digestion. Just distal to the nucleus are found secretory granules giving less intense protein reactions, and of size similar to the others. These likewise do not appear to contain nucleic acid. In cells containing only a few granules, after expulsion, these are small and highly concentrated.

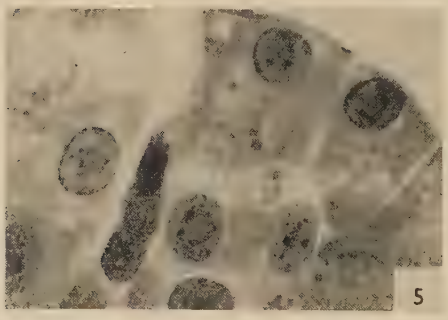
The cytoplasmic structures described above are seen to be contained in a basophilic homogeneous hyaloplasm. This is referred to as the nongranule cytoplasm. It is the site of a high absorption at 254 mμ, reducible by ribonuclease. In the pancreas of this animal, ergastoplasmic striations


1

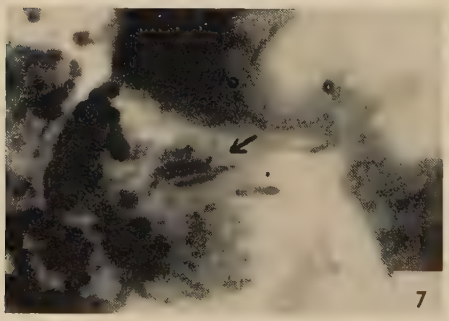

2

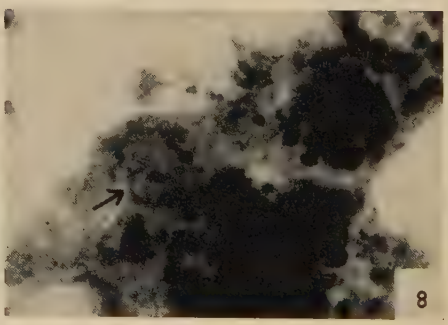

3


4


5

6


7


8

are not apparent, nor does there appear to be a perinuclear ring of especially intense basophilia.

Results of quantitative studies.—Thirty-two pancreases have been completely analysed for cell structure volume, and for cytoplasmic nucleic acid and protein reactions. These stem from both of the experimental situations. The values obtained for the various measured quantities were reproducible, duplicate sets of determinations agreeing to within ten per cent. Variations in the content per cell of secretory granules were considered to represent the stage of the secretory cycle.

A preliminary study was made, using only the Chalkley method, on a series of organs from animals sacrificed at various times during refeeding after 21 days starvation. For this study, the three or four pancreases obtained at each time interval were embedded and sectioned together. Data was obtained for a random sample of each group of organs. Fig. 9 shows the variation in volume of cell constituents during the time of the experiment. A considerable increase in the granule content was indicated very soon after refeeding was begun. Therefore, another group of animals was subjected to the same conditions, and prepared for complete study, both for volume determination, and cytophotometric measurement. Since, in this group, each organ was studied separately, a surprising amount of individual variability was revealed. Table I shows the data obtained for the volumes of the cell constituents of the individual pancreases, the variability of the secretory granules being the largest. Fig. 10 may be compared with Fig. 9, and indicates that the granule content at the end of the experiment is again high. However, values as high occur at only one-half hour after the beginning of the experiment. In both series, the starved condition of the animals was verified by the presence of mitochondria of the rod-

---

Fig. 1–6. An area of a single section of the tadpole pancreas, 390 ×. Frozen and dried. Examined successively by the techniques indicated.
Fig. 1. Phase contrast in oil n_D 1.460.
Fig. 2. Stained with naphthol yellow S to demonstrate protein basic groups.
Fig. 3. Mounted in glycerin and photographed at 254 mμ, near the peak absorption of nucleotides.
Fig. 4. Stained with azure B under conditions specific for the nucleic acids.
Fig. 5. Mounted in glycerin and photographed at 254 mμ, after digestion with ribonuclease. Photographic technique same as Fig. 3.
Fig. 6. Stained with azure B, after digestion with ribonuclease. Photographic technique same as Fig. 4.
Fig. 7. Pancreas 154, from an animal fasted 21 days. Frozen dried, azure and acid fuchsin, 650 ×. Rod-like mitochondria.
Fig. 8. Pancreas 159, from an animal fasted 21 days, and then allowed to feed for one-half hour. Technique as in Fig. 7. Filamentous mitochondria.

type in all animals obtained before the second hour of feeding, high granule contents thus occurring in animals otherwise giving evidence of starvation.

Inasmuch as feeding after prolonged starvation failed to provide experimental control of the secretory cycle, a more drastic stimulus was sought. Babkin and his coworkers [2] have found that the instillation of peptone solution into the mammalian duodenum causes a large scale secretion of pancreatic digestive enzymes, and a corresponding loss of granules from the acinar cells. This stimulus was applied to tadpoles fasted for twenty-four hours, peptone-fed animals being contrasted with unfed controls. In such an experiment, it was expected that organs from peptone-fed animals would show a rapid loss of granules; while the controls, in the absence of a stimulus, would maintain a high granule content. Table II shows the

TABLE I

Volumes of acinar cell constituents during feeding after 21 days fasting.

Time after beginning of feeding (hrs.)	Pancreas	Non-granule cytoplasm		Granules		Nucleolus	
		Mean	Std. error	Mean	Std. error	Mean	Std. error
0	154	1192	45	322	17	14	2
	156	1066	32	232	9	25	2
	157	963	40	261	14	19	2
1/2	158	954	38	405	21	16	2
	159	973	29	453	18	16	2
	160	948	33	332	18	20	2
	161	966	26	316	12	20	2
1	162	780	37	169	9	21	2
	163	785	23	250	11	17	2
	164	1031	27	232	11	20	2
	165	987	24	224	8	17	2
4	166	1029	38	252	11	20	2
	167	1142	49	259	15	19	2
	168	1058	45	86	8	24	2
	169	917	28	267	16	19	2
16	170	838	26	443	18	20	2
	172	1037	45	389	19	22	3
	173	985	45	314	18	20	3

Mean volumes in cubic micra, relative to the standard mean nuclear volume of 200 cubic micra.

results of such an experiment. While a decreasing trend of granule content with time may be discerned in the peptone-fed animals, the controls show an apparent increase of greater magnitude. Thus, on the basis of both the starvation and peptone-feeding experiments, it must be concluded that the physiological control of pancreatic secretion in the tadpole must differ from that encountered in the mammal.

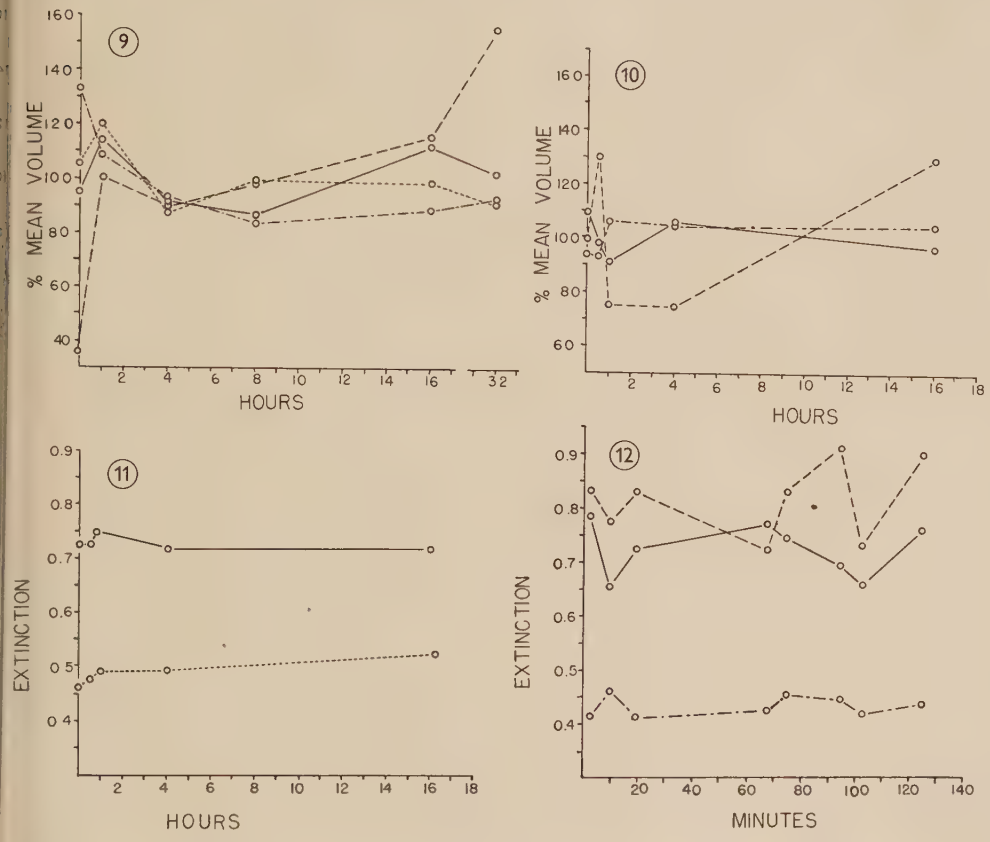


Fig. 9. Variation in the volumes of the cellular constituents during feeding after 21 days' fasting. Nucleus – – – –, Nucleolus –·–·–·–, Non-granule cytoplasm ————, and Secretory granules — — — — —. Each point the average of 3 or 4 organs. (First series.)

Fig. 10. Variation in the volumes of the cellular constituents during feeding after 21 days fasting. Curves designated as in Fig. 9. (Second series.)

Fig. 11. Cytoplasmic absorption of sections as a function of time since the start of feeding, after fasting for 21 days. Upper curve E_{546}, after azure B. Lower curve E_{435}, after naphthol yellow S. Each point the average of three or four animals.

Fig. 12. Cytoplasmic absorption of sections from unfed animals. Azure B, E_{546}: ————, naphthol yellow S, E_{435}, –·–·–·– total ultraviolet absorption, E_{254}, – – – – –. Each point for one animal.

Cytophotometric studies on these same organs were made to determine whether cytoplasmic nucleic acid and protein concentration were similarly independent of the animals feeding behavior. Tables III and IV summarize the results of the measurements. In animals fed after prolonged starvation, it is apparent from Fig. 11 that there is no striking change in the cytoplasm as a function of time, either in basic or acid staining. In the study of the sections from the peptone-feeding experiment, the absorption of the sections at 254 mμ was used as a check on the azure B staining. There is an apparent increase in azure B binding in the peptone-fed animals with a maximum at 75 minutes, which is crudely paralleled by the absorption at 254 mμ (Fig. 12). However, the controls show a random variation in apparent nucleic acid concentration that is almost as great (Fig. 13). The concentration of naphthol yellow S is more constant with time.

However, it may be supposed that the shrinkage or swelling to which the organs are subjected during the processes of fixation, etc., might pro-

TABLE II

Volumes of acinar cell constituents during feeding on peptone and agar, compared to unfed controls.

Series	Minutes from start of feeding	Non-granule cytoplasm		Granules		Nucleolus	
		Mean	Std. error	Mean	Std. error	Mean	Std. error
Peptone fed	15	602	19	236	13	15	3
	32	946	29	416	15	18	1
	40	884	41	340	22	12	2
	71	770	31	364	19	11	2
	99	724	25	214	10	13	2
	133	868	30	290	11	14	2
Unfed	3	820	27	238	10	21	3
	10	788	25	356	14	11	2
	20	834	25	142	7	14	1
	68	890	52	214	15	13	2
	75	952	28	256	16	14	2
	95	1064	46	256	13	15	2
	103	1246	69	336	28	18	2
	125	730	29	492	19	14	1

Mean volumes in cubic micra, relative to the standard mean nuclear volume of 200 cubic micra.

duce a spurious variation in concentration in the finished preparation. If the amount of material per cell is used as the basis for comparison, such effects would be minimized. Further, changes in the amount of material might occur as an addition to the amount of cytoplasm, rather than as concentration changes. Therefore, the amount of absorbing material per cell has been calculated, using as a volume term the non-granule cytoplasmic volume determined by the Chalkley technique. Since cytoplasmic nucleic acid appears to be confined to this structure, and since the measuring technique has been held comparable to the Chalkley procedure, this calculation should be a valid approximation. Fig. 14 illustrates the variation in

TABLE III

Results of photometric measurement of protein and nucleic acid reactions in non-granule cytoplasm of acinar cells. Feeding after 21 days fasting.

Hours from start of feeding	Pancreas	Azure B			Naphthol yellow S		
		Relative concentration[1]	Std. error	Amount per cell[2]	Relative concentration[1]	Std. error	Amount per cell[2]
0	154	0.741	0.027	88	0.462	0.020	58
	156	0.745	0.016	79	0.534	0.023	56
	157	0.691	0.027	66	0.394	0.014	38
1/2	158	0.695	0.027	66	0.488	0.022	46
	159	0.746	0.029	72	0.604	0.021	58
	160	0.806	0.018	76	0.509	0.014	48
	161	0.653	0.027	63	0.304	0.010	29
1	162	0.711	0.024	55	0.477	0.015	37
	163	0.741	0.027	58	0.470	0.012	37
	164	0.806	0.023	58	0.485	0.016	49
	165	0.737	0.025	73	0.531	0.013	52
4	166	0.663	0.018	68	0.501	0.020	52
	167	0.742	0.025	85	0.488	0.021	55
	168	0.646	0.017	58	0.468	0.013	50
	169	0.817	0.020	75	0.510	0.012	46
16	170	0.693	0.024	58	0.578	0.014	48
	172	0.672	0.020	70	0.488	0.015	50
	173	0.788	0.023	78	0.508	0.019	50

[1] Mean extinction (thirty measurements) or 5 μ sections, in areas of non-granule cytoplasm.
[2] 1/10 (Rel. Conc.) (Volume non-granulae cytoplasm) = Amount per cell.

amounts of azure B and naphthol yellow S in the animals fed after prolonged fasting. The decrease, with a minimum at one hour in both constituents is to be interpreted as a decrease in non-granule cytoplasmic volume, rather than a concentration change. During the experiment as a whole, however, there is no overall increase or decrease. In the peptone-fed animals, on the other hand, an apparent increase occurs (Fig. 15). In the unfed controls, where conditions might be expected to remain unchanged, there is an apparent increase of considerable magnitude, a function primarily of the increase in non-granule cytoplasm volume, as shown in Fig. 16.

The volume per cell of zymogen granules may itself be used as an index

TABLE IV

Results of photometric measurement of protein and nucleic acid reactions in non-granule cytoplasm of acinar cells. Feeding on peptone and agar, compared to unfed controls.

Series	Minutes from start of feeding	Azure B			Naphthol yellow S			U.V. absorption		
		Relative concentration[1]	Std. error	Amount per cell[4]	Relative concentration[2]	Std. error	Amount per cell[4]	Relative concentration[3]	Std. error	Amount per cell[4]
Peptone fed	15	0.549	0.018	33	0.396	0.011	25	0.773	0.017	47
	32	0.632	0.017	60	0.398	0.010	38	0.752	0.024	71
	40	0.685	0.015	60	0.386	0.011	34	0.828	0.019	73
	71	0.855	0.023	66	0.455	0.011	35	0.892	0.016	69
	99	0.770	0.024	56	0.405	0.010	29	0.860	0.017	62
	133	0.740	0.020	58	0.419	0.013	36	0.825	0.030	72
Unfed	3	0.788	0.020	65	0.415	0.012	34	0.831	0.014	68
	10	0.654	0.023	52	0.561	0.014	36	0.775	0.019	61
	20	0.732	0.020	60	0.410	0.012	34	0.831	0.023	69
	68	0.770	0.025	68	0.422	0.012	37	0.722	0.019	64
	75	0.744	0.021	71	0.452	0.014	43	0.835	0.028	79
	95	0.693	0.017	74	0.445	0.016	47	0.911	0.030	97
	103	0.658	0.021	82	0.418	0.012	52	0.732	0.025	92
	125	0.758	0.032	55	0.434	0.015	32	0.907	0.036	66

[1] Mean extinction at 546 mμ, 5 μ sections, 20 measurements per pancreas.
[2] Mean extinction at 435 mμ, 4 μ sections, 20 measurements per pancreas.
[3] Mean extinction at 254 mμ, 4 μ sections, 20 measurements per pancreas.
[4] 1/10 (Rel. Conc.) (Volume non-granulae cytoplasm.)

of secretory activity; and the correlation of this quantity with the other measured cell characteristics may be studied. Table V indicates the relation between granule content and a number of the parameters determined. It will be seen that only the total cytoplasmic volume shows significant correlation with granule volume. The correlation between ultraviolet absorption and basic staining is positive and significant, showing that the two methods are in crude agreement.

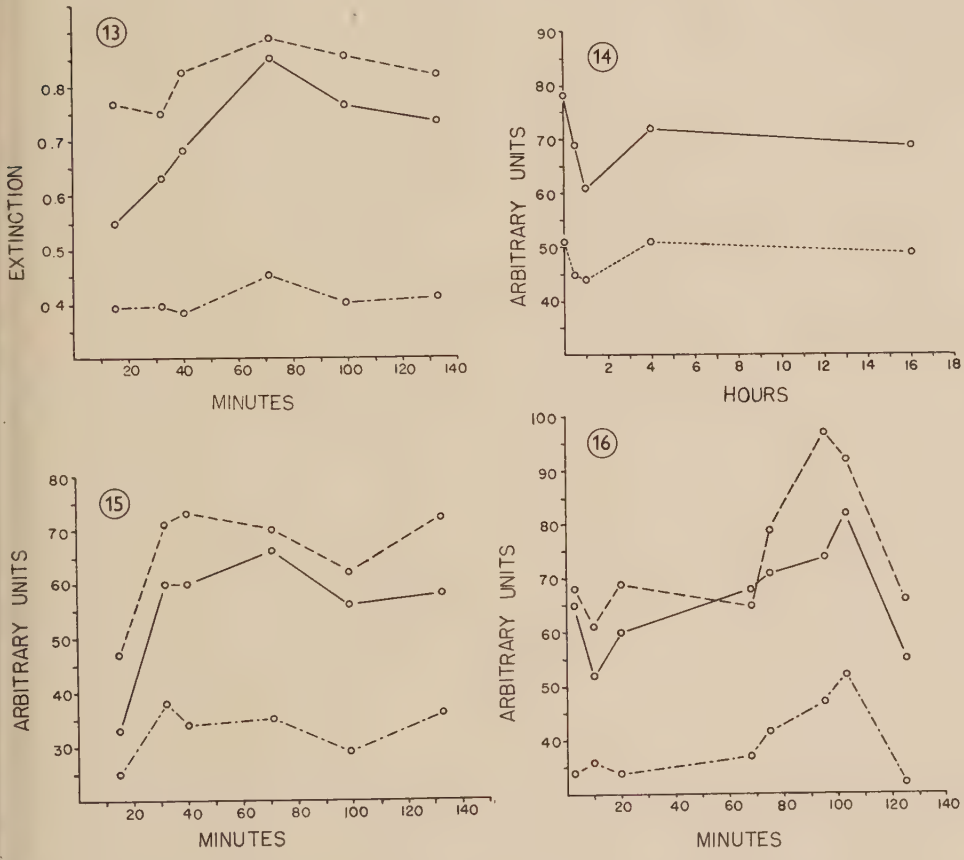


Fig. 13. Cytoplasmic absorption of sections from animals fed peptone and agar for the times indicated. Curves designated as in Fig. 12. Each point for one animal.
Fig. 14. Amount per cell, in arbitrary units, of azure B (upper curve), and naphthol yellow S (lower curve) bound by non-granule cytoplasm during feeding after fasting for 21 days. Each point the average for three or four animals.
Fig. 15. Amount per cell, in arbitrary units, of azure B ————, naphthol yellow S –·–·–·–, and total absorption at 254 mμ – – – –, during feeding on peptone and agar. Individual animals.
Fig. 16. Amount per cell, in arbitrary units, of the same reactions as in Fig. 15. Same curve designation. Individual animals. Unfed controls.

DISCUSSION

The picture thus obtained of the volumes of secretory cell structures, and of the absorption of the cytoplasm after specific staining techniques, is complicated by a striking variation from individual to individual. However, numerous authors [3, 24] have nevertheless implied the secretory stage of a pancreas cell from the content of secretory granules. When such detailed studies have been made, particularly with reference to the Golgi material, other changes are found to correlate with the granule content. Together with the controlled studies on mammalian forms, the evidence for a cyclical synthesis of zymogen granules is quite good [2, 14, 17]. Thus, if the synthesis taking place in this form is cyclical, as in other vertebrates; the variation found in granule contents of acinar cells, irrespective of the experimental conditions, indicates that the secretory cycle is taking place independently in each animal. The uniformity of granule content throughout the pancreas is striking and would also indicate that the granule content is not random. Thus, the granule content itself may be used as an index of the secretory activity of the cell. Since the expulsion phase is a rapid one, intermediate values should represent glands in the process of restitution, while the higher values represent replete organs. Glands having just expelled their granules should be then those with the lowest values. On this assumption, a relation of any characteristic to the secretory cycle should be detectable by its correlation with the granule content of the cells.

Since the parameters indicated in Table V are independent of granule content, it must be concluded that they do not vary as a result of secretory activity. It therefore appears probable that the variability encountered represents differences due to factors other than those involved in the secretory cycle. While the animals used here are derived from a single years hatch of eggs, the variation in size presumably indicates differences, either ecological or physiological, among the animals. However, there is not a significant relation between cell size (granules plus non-granule cytoplasm) and body length of the animals.

This study does not indicate, therefore, that either the basophilia or the ultraviolet absorption give indications of any major change in cytoplasmic nucleic acid as a function of the activity of the pancreatic exocrine cells. Non-granule protein of the cytoplasm is similarly independent of the secretory cycle.

On the basis of the data presented in the biochemical study by Daly and Mirsky [11], it appears that the dry weight per cell remains constant

throughout the secretory cycle. In view of the fact that the amount of enzyme protein was shown to increase during the experiments, they concluded that a non-enzymatic protein was synthesized at the same time as the granules were extruded. If this relationship also holds for the tadpole, then the amount of non-granule protein should show an inverse relation to the granule content. However, the amount of non-granule cytoplasmic protein does

TABLE V

Values of the correlation coefficient (r) and of the normal deviate of z[1] for the indicated relations.

Correlation of	With	Number of cases	r	$z\sqrt{n'-3}$
Nuclear volume	Granule volume	6	+0.057	0.18
Nucleolar volume	,, ,,	32	−0.219	1.18
Non-granule cytoplasm volume	,, ,,	32	−0.023	0.12
Concentration of azure B . .	,, ,,	32	−0.0001	0.0006
Extinction at 254 mμ	,, ,,	14	+0.123	0.41
Concentration of naphthol yellow S	,, ,,	32	+0.242	1.30
Total cytoplasmic volume . .	,, ,,	32	+0.537	3.23[2]
Extinction at 254 mμ . . .	Concentration of azure B	14	+0.507	1.769[2]

[1] According to R. A. Fisher, *Statistical Methods for Research Workers*, London, 1936, p. 205.
[2] Indicates a significant difference from $r = 0$.

not appear to have any relation to the granule content. On the contrary, the results obtained here are consistent with the view that the amount of ground cytoplasm, containing a high concentration of nucleic acid and protein remains constant during the secretory cycle. The amount of this ground cytoplasm is possibly a function of other factors, such as the nutritional condition of the animal. The granules are formed as an addition to this basic structure, and thus the total cytoplasmic volume in cells with high granule contents exceeds that of those which are depleted of granules ($r = +0.54$). It should be appreciated that the concentration of protein in the granules is probably many times that of the non-granule cytoplasm.

Thus the conclusion from this study in regard to the nucleic acid relation to the enzyme synthesis taking place is in accord with the biochemical results. Previous microscopic studies on the pancreas have not combined quantitative measurement with integration for volume. The dilution of the

absorbing non-granule cytoplasm by non-absorbing zymogen granules can lead to error in estimating concentration, either by objective or subjective methods. This is particularly true in the mammalian pancreas, where the proportions of non-granule to granule cytoplasm are quite different from those in the tadpole. The basal part of the cell contains few granules at any time in the tadpole. There is thus a convenient free area through which concentration measurements may be made. The assumption is made that the non-granule cytoplasm in the interstices between the zymogen granules has the same concentration of absorbing substance as does that in the basal portion. In any case, this material must represent but a negligible fraction of the total non-granule cytoplasmic volume.

Recent studies on the rate of incorporation of P^{32} into PNA phosphorus of gland cells [13] indicate that nucleic acid turnover is not a necessary concomitant of protein production. In the pancreas, as well as in the oviduct of the laying hen, it was found that low rates of P^{32} incorporation occurred during secretion. In feather regeneration, where there is a continual replacement of whole cells, a high rate of PNA synthesis is found. During the course of lactation, the mammary gland exhibits an increasing content of PNA, which falls with weaning [21]. Since the lumen of the active mammary gland, and the milk itself, contain portions of the ground cytoplasm of the epithelial cells [19], this must indicate a continual replacement of cellular PNA.

The observed differences in the behavior of the nucleic acids can be attributed to the modes of secretion involved. Where entire cells are lost in the secretory process (holocrine secretion) it is obvious that a high rate of PNA synthesis must be maintained. In merocrine secretion, the portions of the glandular epithelial cells nearest the lumen are lost into the secretion. The mammary gland is the classical example of this type. Thus the PNA synthesis found during secretion in these two types must be considered as a special case of cell growth, associated with restoration of ground cytoplasm. In the more specialized apocrine secretory cells, e.g. pancreatic acinar cells, or the serous cells of the hen's oviduct, protein synthesis may go on apart from a concomitant synthesis of nucleic acid. Thus, as has been pointed out [13], the number of possible views of the relation of PNA to protein synthesis is somewhat reduced. From this viewpoint, if PNA is concerned with protein synthesis in the pancreas, it must function as a catalyst or template. It may thus be an essential component of an organoid which carries on the actual synthesis [16].

A wide variety of apocrine secretory cells are characterized by the pres-

ence of secretory granules, corresponding to the description above of the granules in the pancreatic acinar cells. Similar granules are found, for example, in the epithelial cells of the protein-producing region of the oviduct in the hen [29]. These structures may be of significance as the site of a "packaging" process in protein synthesis. That is, the accumulation of the synthesized protein in these structures may serve to deplete the PNA-containing structures of the ground cytoplasm, and thus maintain an equilibrium condition which favors synthesis. There is some recent support for this view in the facts that (a) the protein of the microsomal nucleoprotein of pancreas cells can be shown to incorporate N^{15}-glycine more rapidly when secretion is stimulated [1], while (b) the major site of localization of pancreatic secretory enzymes appears to be in the zymogen granules (23). In the mammary gland, while fat droplets are prominent, zymogen granules of the typical sort are not found [19]. Thus, the type of relation observed between PNA synthesis and the overall rate of protein production in different cell varieties may be a function of the presence or absence of the "packaging" phase.

SUMMARY

1) The cytochemical examination of frozen-dried preparations of the pancreas of the *Rana clamitans* tadpole leads to the following conclusions:

a) The zymogen granules contain a high concentration of protein, while no nucleic acid is detectable.

b) The mitochondria contain a higher concentration of protein than the surrounding cytoplasm, while the nucleic acid concentration appears to be lower.

c) The nucleolus-associated chromatin appears to show a higher ratio of PNA to DNA than other chromatic bodies of the nucleus.

2) Volumetric study by the Chalkley technique indicates that the secretory process consists of the addition of the zymogen granules to an amount of ground cytoplasm that is not a function of the secretory cycle. The volumes of the nucleus and of the nucleolus are independent of the secretory stage.

3) The amount per cell and concentration of cytoplasmic nucleic acid, as measured both by basic staining and ultraviolet absorption, is not correlated with the secretory cycle.

4) The amount per cell and concentration of cytoplasmic protein, as measured by acid dye binding, is also independent of the secretory cycle.

5) The stability of cytoplasmic nucleic acid in protein-producing gland cells of the apocrine type as contrasted with growing cells and other types of gland cells is considered to be associated with the presence of zymogen granules. These structures may function in removing synthesized protein from the site of the synthetic reaction.

I am indebted to Professor A. W. Pollister for the interest he has had in this work, and for the advice and guidance so generously extended. To Arline Deitch, Marion Himes, Rachel McMaster, and Leonard Ornstein I am grateful for stimulating discussion and suggestions. Liselotte Mezger-Freed has offered constant encouragement and invaluable assistance.

I should like to thank Dr. Jack Schultz for his criticism of the manuscript.

REFERENCES

1. Allfrey, V., Daly, M. M., and Mirsky, A. E., *J. Gen. Physiol.* **37**, 157 (1953).
2. Babkin, B. P., The Secretory Mechanism of the Digestive Glands. New York, 1950.
3. Bowen, R. H., *Quart. Rev. Biol.* **4**, 299 (1929).
4. Brachet, J., *Compt. Rend. Soc. Biol.* **133**, 88 (1940).
5. —— Embryologie chimique. Masson et C^ie, 1st ed., Paris, 1945.
6. —— Le rôle des acides nucléiques dans la vie de la cellule et de l'embryon. Masson et C^ie, Paris, 1952.
7. Caspersson, T. O., Cell Growth and Cell Function. W. W. Norton and Co., 1st ed., New York, 1950.
8. Caspersson, T. O. and Schultz, J., *Nature* **143**, 602 (1939).
9. Caspersson, T. O., Landström–Hyden, H., and Aquilonius, L., *Chromosoma* **2**, 111 (1941).
10. Chalkley, H. W., *J. Natl. Cancer Inst.* **4**, 47 (1945).
11. Daly, M. M. and Mirsky, A. E., *J. Gen. Physiol.* **36**, 243 (1952).
12. Deitch, A. D., *Anat. Rec.* **117**, 583 (1953).
13. Deken–Grenson, M. de, *Biochim. et Biophys. Acta* **12**, 560 (1953).
14. Duthie, E. S., *Proc. Roy. Soc. London B* **114**, 20 (1934).
15. Flax, M. A. and Himes, M. H., *Physiol. Zoöl.* **25**, 297 (1952).
16. Freed, J. J., To be published.
17. Hirsch, G. C., *Z. Zellforsch. u. mikroskop. Anat.* **15**, 36 (1932).
18. Hokin, L. E. and Hokin, M. R., *Biochim. et Biophys. Acta* **11**, 591 (1953).
19. Jeffers, R. R., *Am. J. Anat.* **56**, 257 (1935).
20. Kauffmann, B. P., McDonald, M. R., Gay, H., Wilson, K., and Wyman, R., *Carnegie Inst. Washington Yr.Book* **46**, 136 (1947).
21. Kirkham, W. R. and Turner, C. W., *Proc. Soc. Exptl. Biol. Med.* **83**, 123 (1953).
22. Malmgren, B. and Heden, C.-G., *Acta. Pathol. Microbiol. Scand.* **24**, 417 (1947).
23. Marshall, J. M., *Exptl. Cell Research* **6**, 240 (1953).
24. Nassonow, D., *Arch. mikroskop. Anat. u. Entwicklungsmech.* **97**, 136 (1923).
25. Noback, C. R. and Montagna, W., *Am. J. Anat.* **81**, 343 (1947).
26. Ornstein, L. and Pollister, A. W., *Trans. N.Y. Acad. Sci.* **14**, 194 (1952).
27. Pollister, A. W., *Lab. Investigation* **1**, 106 (1952).
28. Rabinovitch, M., Valeri, V., Rothschild, H. A., Camara, S., and Junqueira, L.C.U., *J. Biol. Chem.* **198**, 815 (1952).
29. Richardson, K. C., *Phil. Trans. Roy. Soc. London B* **225**, 149 (1935).
30. Ries, E., *Z. Zellforsch. u. mikroskop. Anat.* **22**, 523 (1935).
31. Swift, H. H., *Physiol. Zool.* **23**, 169 (1950).
32. van Weel, P. B. and Engel, C., *Z. vergleich. Physiol.* **26**, 67 (1938).

Experimental Cell Research, **9**, *35–41 (1955)*

AN IMPROVED METHOD FOR CULTIVATING FIBROBLASTS OF THE NEWT

J. BOSS

Department of Anatomy, Middlesex Hospital Medical School, London, England[1]

Received September 11, 1954

As has already been reported [5, 10, 12] the large size of the cells makes cultures of newt tissues more favourable for cytological investigation than those of the tissues of warm-blooded animals. On the other hand, their slow and very variable growth has hitherto rendered amphibian tissue cultures much less suitable for quantitative work than the more easily standardised, quickly growing cultures of avian and mammalian tissues.

This technical difficulty has now been overcome, and in this paper a procedure is described whereby rapidly growing, mitotically active cultures of newt fibroblasts can be regularly obtained. This material should be very favourable for investigating such problems as the effects, both qualitative and quantitative, of chemical and physical agents on mitosis.

METHODS

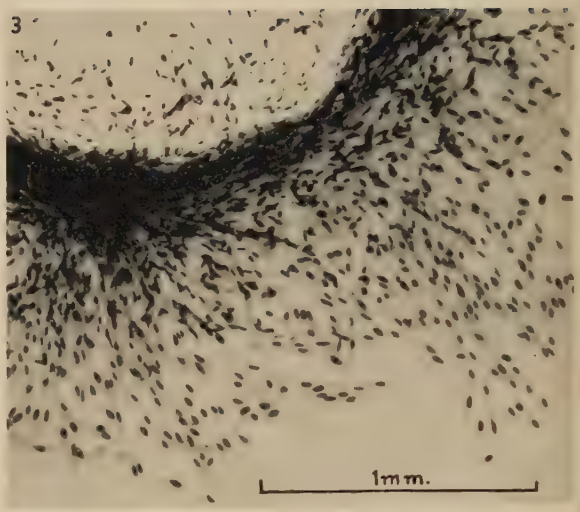
The individual operations used in the present studies are the same as those already described in detail and discussed [5]. In those earlier studies, however, a number of different tissues and methods were used, whereas further experience showed that the cultures obtained by explanting the coracoid cartilage were by far the most satisfactory for cytological investigations, and that larger and more standardised cultures could be grown if the methods previously described were combined in the following procedure.

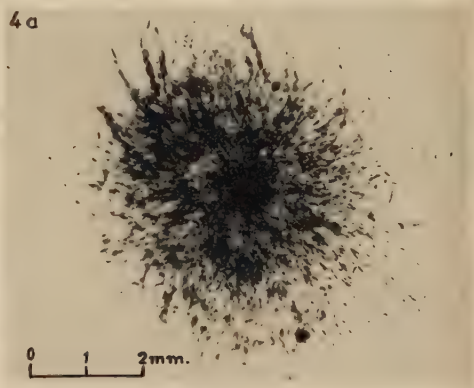
Whole coracoid cartilages of the Adriatic crested newt (*Triturus cristatus carnifex*) were explanted, each being put into a hanging drop of a mixture of 11-day chick-embryo extract and cockerel's plasma. The cultures were replenished weekly by Maximow's method [5] for 3 or 4 weeks; there was then a broad growth of fibroblasts, probably of perichondrial origin [4], suitable for studies on individual cells, both living and fixed. A culture with the outgrowth and original explant *in situ* was said to be at *Stage I* (Fig. 1).

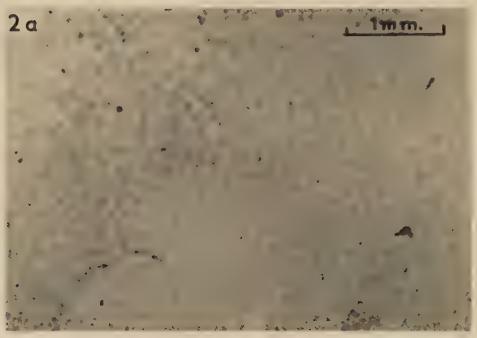
The cartilage explant was now removed, leaving the outgrowth still intact in the medium. This was done by pressing the tip of a blunt probe on to the cartilage, and

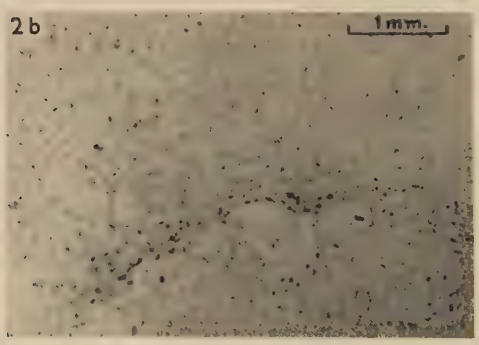
[1] Present address: Strangeways Research Laboratory, Cambridge, England.

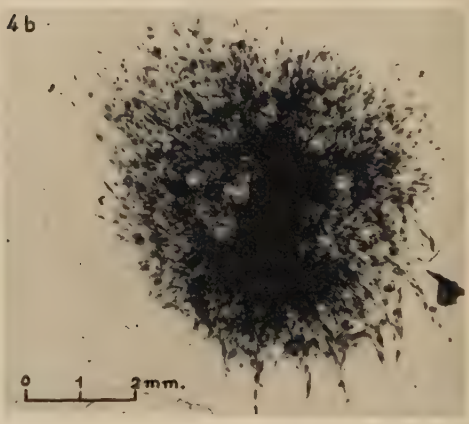
1
1mm.


3
1mm.


4a
0 1 2mm.


2a
1mm.


2b
1mm.


4b
0 1 2mm.

agitating the latter with small excursions from side to side to disengage it from the clot. The freed cartilage was explanted again to begin Stage I of another culture. The cultivation of the outgrowth left *in situ* was continued by Maximow's method, and the tissues now grew, not only centrifugally, but also centripetally into the space left by the explant (Fig. 2). The outgrowth was replenished as soon as the explant has been removed, and after 2 or 3 more weekly replenishments, there was a considerable thickening of the zone of cells which previously had been adjacent to the explant (Fig. 3). Cultures from which the explant had been removed, but the outgrowth of which was still *in situ*, were described as being at *Stage II*.

The thickened zone was then excised as a ring, which was cut open at one place. The resulting band was divided into 3, 4, or 5 pieces, each of which was explanted separately into a drop of fresh medium. In each of the preparations so made the explanted fragment of the Stage II culture was about 0.3 mm in diameter, there having been considerable retraction of the surface of the fragment on excision and division. The new preparations, termed *Stage III* cultures, were replenished once a week by Maximow's method for about 3 weeks, at the end of which time each culture was 5–6 mm in diameter (Fig. 4). During the middle of the third week of Stage III, centrifugal expansion diminished, while the central mass of cells became thicker and enlarged irregularly; cultures such as that depicted in Fig. 4b developed in this way.

After 3 weeks of replenishment a Stage III culture was transplanted, a central portion with a diameter about one-half that of the whole culture being excised from the medium. The excised piece could be divided to give 2, 3, or 4 daughter cultures; these were replenished weekly by Maximow's method and were again transplanted and divided after 3 weeks. Thus growth could be maintained by transplantation every 3 weeks with intervening weekly replenishments, and the number of cultures increased at each transplantation. Cultures have not yet been taken beyond the third passage of Stage III, but it is probable that they could be maintained indefinitely in the way described.

During the present studies 88 cultures, derived from 55 original explants of

---

Fig. 1. Culture at Stage I. Part of the explant of coracoid cartilage is seen as a dark segment in the upper left-hand corner. The remainder of the field is filled with fibroblasts. The culture was fixed two weeks after explantation. Fixed in Methanol; May-Grünwald and Giemsa.

Fig. 2. Figures 2a and 2b show similar preparations. Cultures fixed 36 hours after removal of the explant and replenishment of the outgrowth, i.e. the cultures are in the early part of Stage II. A line of more closely aggregated cells marks the limit of where the explant lay; both photographs show part of a culture with its periphery towards the upper part of the picture. There is little overlap between cells and virtually none between nuclei. Maximow's fixative; Feulgen.

Fig. 3. A culture after 3 days in Stage II. The appearance is really typical of the third week of this stage, but the culture was allowed to develop a thick outgrowth in Stage I; usually the explant was removed while the outgrowth was one cell deep. There is a thick mass of cells demarcating the region previously occupied by the explant, a part of which lay in the less cellular area shown in the upper left-hand part of the figure. Fixed in methanol; May-Grünwald and Giemsa.

Fig. 4. Two cultures from the same batch after 4 weeks in the second passage of Stage III, showing the narrow range of variation in growth. — (a) The fragment transplanted at the beginning of the passage had a diameter about one-third that of the central black spot in the figure. (b) Another culture. The mass formed by the innermost cells is extensive and irregular in outline. Maximow's fixative; iron haematoxylin.

coracoid cartilage, were grown; in addition, this account is based also upon unpublished observations made in the course of work already reported [1, 2, 3, 4, 5, 6, 7, 8]. Of the 88 cultures, 12 were maintained beyond the first passage of Stage III.

BEHAVIOUR OF CULTURES

The types of cell present in cultures of coracoid cartilage are fibroblasts and macrophages, but the latter are uncommon at all stages, provided that no bone or muscle is included in the explant.

The uses of the Stage I culture have been illustrated by earlier work [6, 7, 8]. An explant of coracoid cartilage yielded an outgrowth well suited for studies on mitosis in both fixed and living material, although the optical conditions for the examination of living cells by phase-contrast microscopy were best near the periphery of the outgrowth.

During the first week of Stage II, the cells grew so as to form a single layer (Fig. 2) in a plane near the cover slip. Between 100 and 200 mitotic figures could be found in a culture fixed in late Stage I or early Stage II. The peculiar usefulness of cultures in the first week of Stage II was due to the single-layered arrangement of cells, which provided good optical conditions, particularly for phase-contrast microscopy; a second advantage was that the cells could be counted directly, so that their total number in a culture could be ascertained. As already mentioned, in cultures at Stage II the cells eventually formed several layers with an especially thick zone immediately outside the former boundary between explant and outgrowth (Fig. 3).

In Stage III the explant was small, and in its neighbourhood the level surface of the medium was disturbed much less than it had been near the coracoid cartilage in Stage I. In consequence, the whole of the outgrowth could be viewed under good optical conditions during the first week. Later the cells lay so densely in the central region that phase-contrast microscopy had to be restricted to the outer zone; since the area of the whole culture was enlarging, however, the number of cells available for examination in this way steadily increased until the middle of the third week of Stage III, when expansion in area diminished and the greater part of the culture became too thick for detailed observation.

Cultures at Stage III differed from other types of amphibian tissue cultures in the following characteristics. The latency of the outgrowth was brief; the first protoplasmic processes to project from the explant were visible within twelve hours, and after two days there was a zone of many fibroblasts lying free of the explant; mitosis was present even among the earliest cells to migrate from the explanted fragment. Fibroblasts may emerge from an

original explant of newt lung after almost as short a delay, but, in the author's experience, no other cultures of amphibian tissue have such a constant latent period as those of the cells of coracoid cartilage at Stage III.

For amphibian fibroblasts, the rate of growth was rapid, an increase in diameter of 2 or 3 mm per week being usual for the cultures during the first two weeks of Stage III. This rate is equalled by some cultures of newt epithelium which, however, have but few mitotic cells in the zone of growth at one time, and have other disadvantages for cytological research [4]. Just as the latency of Stage III cultures was very constant, so was their growth-rate; Figs. 4a and b illustrate the narrow range of variation in area among cultures explanted from the same newt, treated in the same way throughout and fixed at the same time. While cultures of fibroblasts from the embryonic chick usually grow in this regular way, the rate of growth of newt tissues *in vitro* is generally very variable, as already mentioned.

The number of cells simultaneously in mitosis in cultures at Stage III is comparable with that at the two earlier stages, but is probably less variable. At the middle of the second week of Stage III, i.e. about three days after the first weekly replenishment following Stage II, there were about 100 cells simultaneously in division but the number of cultures fixed in this week is insufficient for a more precise estimate, and counts made on living cultures are unreliable except for rough guidance, although the number of mitotic cells in a living culture of newt fibroblasts is much more readily estimated than in living cultures of avian and mammalian tissues. During the third week of Stage III, while the central region of the culture continued to increase in thickness, the number of mitotic figures among the more easily observable cells of the outgrowth rapidly fell.

All these features are exhibited whether the cultures are prepared by explanting a fragment of an outgrowth or by transplanting the whole or a part of a Stage III culture which has been maintained by Maximow's method for about three weeks. The behaviour and appearance of a Stage III culture is the same whether it is examined at the first or later passages.

DISCUSSION

Cultures of perichondrial fibroblasts of the newt, at Stage III of the procedure just described, fulfil the conditions of constant behaviour and ample growth which render them suitable for quantitative investigations. At the same time direct observations may be made on individual cells, for which purpose, as already pointed out, amphibian tissues in culture are much more

suitable than those of warm-blooded animals, hitherto the only metazoan type to be adopted for quantitative studies *in vitro*.

For example, cultures of chick tissues have been widely used to investigate quantitatively the effect of radiations on the extent of the outgrowth, on the number of cells in each phase of mitosis and on the incidence of abnormal mitosis and degeneration [14.] The intracellular detail of damage to chick fibroblasts by radiation has also been studied to some extent [14], but such observations are more accurately and conveniently made on the tissues of large-celled organisms, such as those of certain plants [e.g. 11]. The Stage III culture of newt fibroblasts, however, appears to combine in the same material the advantages of chick tissue cultures for quantitative research with those of e.g., the hyacinth or onion for the study of intracellular changes.

Stage III cultures of newt cells should be almost as convenient for chemical analysis as those of chick fibroblasts [e.g. 9], but in newt cells, it would be much easier to correlate gross chemical changes with alterations in intracellular anatomy and cytochemical staining reactions. For chemical analysis it would be desirabe to have a large amount of tissue, and there seems to be no reason why stocks of Stage III cultures could not be maintained and multiplied in roller tubes or Carrel flasks. Maintenance in some such way might also be convenient for providing a stock of comparable material during a series of observations on individual cells. It is not possible to say whether these cultures of newt fibroblasts could be disrupted into cell suspensions and grown as diffuse cultures similar to those already prepared from tissues of the embryonic chick [13].

SUMMARY

1. A technique is described whereby fibroblasts of the newt may be cultivated as abundantly and reliably as cells of embryonic chick tissues.

2. The coracoid cartilage is used and the method of cultivation includes both replenishment by Maximow's method and transplantation.

3. Such cultures are suitable for quantitative investigations as well as for studies on individual cells.

4. Possible uses of these cultures are discussed.

The author is grateful to Dr. Honor B. Fell, F.R.S., for her friendly criticism of the work, and to both her and Professor E. W. Walls for reading the manuscript of this communication. Mr. P. Runnicles assisted with the photography. The apparatus with which these studies were made was almost entirely provided by the Central Research Fund of the University of London and by the Medical Research Council.

REFERENCES

1. Boss, J., *J. Physiol.* **119**, 34*P* (1953).
2. —— *J. Anat.* **87**, 445 (1953).
3. —— *J. Physiol.* **120**, 32*P* (1953).
4. —— Ph. D. Thesis, Cambridge 1953.
5. —— *Exptl. Cell Research* **7**, 215 (1954).
6. —— *ibid.* **7**, 225 (1954).
7. —— *ibid.* **7**, 215 (1954).
8. —— *ibid.* **7**, 443 (1954).
9. Davidson, J. N., Leslie, I., and Waymouth, C., *Biochem. J.* **44**, 5 (1949).
0. Hughes, A. F. W. and Preston, M. M'E., *J. Roy. Microscop. Soc.* **49**, 121 (1949).
1. La Cour, L. F., *Heredity* **6** (Suppl.) 163 (1953).
2. Preston, M. M'E., *J. Roy. Microscop. Soc.* **49**, 65 (1949).
3. Rinaldini, L. M., *Nature* **173**, 1134 (1954).
4. Spear, F. G., in Cade, Sir S., Malignant Diesase and its Treatment by Radium. John Wright, Vol. I, p. 292 ff. 1948.

NEURON DEVELOPMENT FROM TRYPSIN-DISSOCIATED CELLS OF DIFFERENTIATED SPINAL CORD OF THE CHICK EMBRYO[1]

MARGARET W. CAVANAUGH

Department of Zoology[2], University of Chicago, Chicago, Ill., U.S.A.

Received September 14, 1954

Beginning in 1949, a systematic program of investigations was started to study the fate of single-cell suspensions, obtained by the dissociation into their constituent cells of embryonic tissues when introduced into embryos [8, 9]. This was later expanded to correlated observations on the behavior of the reaggregated cell masses on the yolk sac [1] and *in vitro*. In 1952, Moscona and Moscona [2, 3] published independently similar experiments on the development of dissociated and reaggregated cells of chick embryos in tissue culture. Both of these series of experiments attested to the retention of tissue-forming abilities in the dissociated cells, as well as to their ability to reconstitute histologically complexes characteristic of the original cell types.

As this method is useful not only in demonstrating the faculty for collective reorganization of cells in random dispersion, but also in furnishing, conversely, populations of single cells of a specific character, which then can be subject to experimentation as separate individuals, it promises to have wide applicability. For this reason, results obtained with this technique, which vary from tissue to tissue, deserve to be placed on record. The following is a brief description of its application to the embryonic nervous system of the chick.

MATERIAL AND METHODS

Spinal cord of the chick embryos in advanced stages of differentiation up to seven days of incubation age proved most suitable for dissociation. In older embryos,

[1] Supported by grants from the Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago and from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

[2] Present address: Division of Anatomy, University of Tennessee, Memphis 3, Tennessee, U. S. A.

the advanced development of the fiber tracts left an undue amount of extracellular debris after dissociation and only a relatively small proportion of cells seemed to survive the treatment (see below). For best results, therefore, embryos should be used during the first week of incubation.

The embryonic spinal cord was dissected in Tyrode's solution; blood and adhering connective tissue were removed; and the clean fragments were then transferred to an osmotically balanced calcium- and magnesium-free Tyrode solution at pH 7.8, which was renewed several times for washing. Dissociation was then carried out essentially according to the technique described by the Mosconas, with slight modifications. A fresh solution of trypsin, recrystallized and salt-free (Worthington Biochemical Sales Corporation), was prepared with Ca- and Mg-free Tyrode solution in a final concentration of 3:10,000. The tissue fragments were placed in about 1 or 2 cc of this solution in a hollow-ground slide and incubated at 37°C for one hour. We did not find it advantageous to raise the pH of the trypsin solution by adding KOH as recommended by the Mosconas to obtain satisfactory dissociation. In fact, the alkalization, when tried, increased the stickiness of the dissociated cells (see below).

The tissue fragments emerged from this procedure somewhat swollen, with softened consistency and loosened cohesion. They could then be easily broken up into their constituent cells by repeated sucking in and out of a small-bore pipette. The single cell suspension thus obtained was then transferred to about 5 cc of Ca- and Mg-free Tyrode solution in which the cells were spun down by low-speed centrifugation for about two minutes. During the latter part of the trypsin treatment, the cells developed considerable stickiness along their surfaces, hence clung together readily as they were driven together by the centrifugal force. If for any reason, it might be desirable to reduce the stickiness of the cells, this could be accomplished by adding hyaluronidase to the trypsin solution [4]. The dissociated cell masses in their random dispersion will henceforth be referred to as "dispersates".

The loose clumps obtained from centrifugation were then washed with care to avoid further disruption in several changes of Ca-Mg-free Tyrode and then cut into pieces suitable for explantation. The explants were placed in hanging-drop cultures in Maximow slides, prepared with equal amounts of fresh blood plasma and 25 per cent embryo extract.

In a modification of this technique, the clumps obtained after the first centrifugation were again broken up by repeated mechanical comminution through small-bore pipettes. After this second treatment, the cells proved to be less sticky. They could then be washed, centrifuged, separated a third time and, after final washing in suspension in Tyrode solution or 10 per cent embryo extract, plated out in the culture clot (Fig. 1).

The dispersates obtained by these two procedures differ in that the former contains small cell clusters from the start, whereas the latter consists largely of isolated cells dispersed through the culture medium. In a number of cases the latter method was used in an exploratory way to produce artificial mixtures of cell populations of different types. For this purpose, non-nervous tissues (metanephros, muscle, liver) were dissociated in a similar way, the suspensions appropriately mixed with suspensions of nerve cells, then centrifuged and seeded out on plasma clots as described above.

The cultures were reared under standard tissue culture conditions, then fixed in 10 per cent neutral Formalin and impregnated *in toto* according to Bodian's Protargol technique.

RESULTS

The mortality of the dissociated cells seemed to increase with age. Incomplete data indicate that average survival was about 30 to 40 per cent in dispersates from five-day cords, 15 to 20 per cent in those of eight-day cords, and no more than 5 per cent in those from ten- to twelve-day cords.

At no time was there evidence of any tendency of cells to concentrate by an active process of mutual attraction. However, random movements, buffeting and other passive dislocations brought sufficient numbers of cells into mutual chance contact which they then maintained because of their surface stickiness. Primary clusters formed during centrifugation likewise tended to remain aggregated. These clumps tended to assume spherical shape, much like embryonic cleavage stages, which is indicative of a high-surface tension.

The first evidence of nerve fiber sprouts was seen after about five hours of isolation. Examples of the subsequent development of these cultures are given in Figs. 2–10. The following observations are noteworthy.

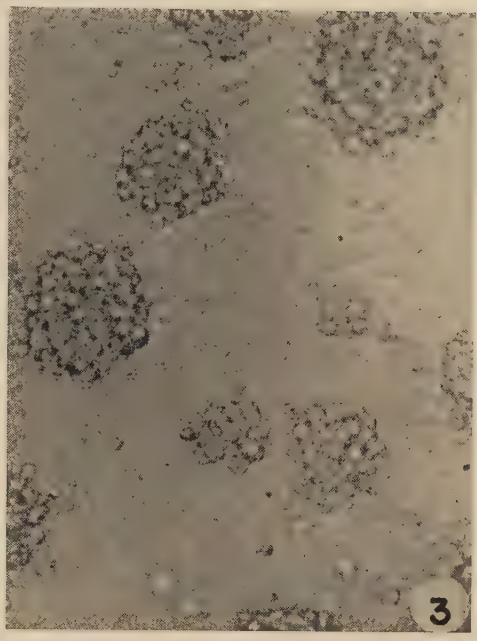
Single nerve cells in complete isolation can give rise to typical naked nerve processes which may reach the length of 3 to 4 mm within as many days. Most of these single neurons are unipolar, with a fair admixture of bipolar ones. Multipolar neurons are relatively scarce. To what extent the different numbers of processes reflect constitutional differences among the neurons on the one hand and properties of the local medium on the other [10], remains undetermined, but it would seem of interest to make a systematic test of neuroblasts from different parts of the embryonic nervous system with regard to this feature.

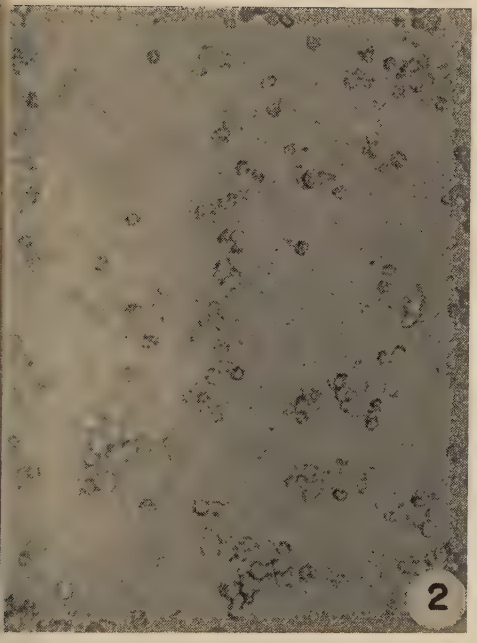
In some cultures, the clumps tended to stay compact, whereas in others they showed a tendency to spread out in the form of sheets, constituting an intricate network of cells and fibers covering the surface of the clot. These

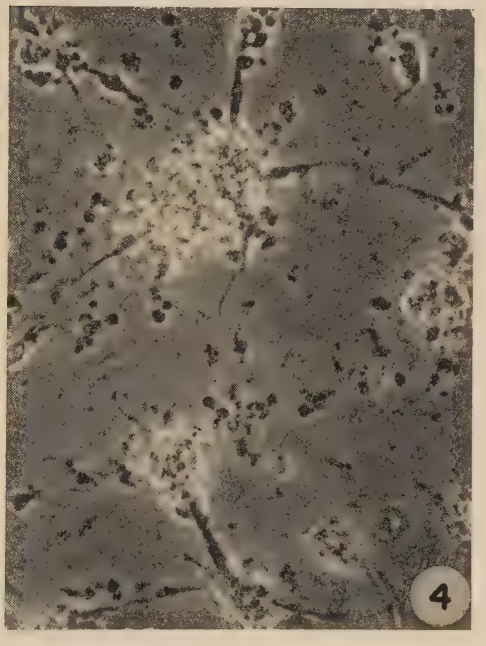
---

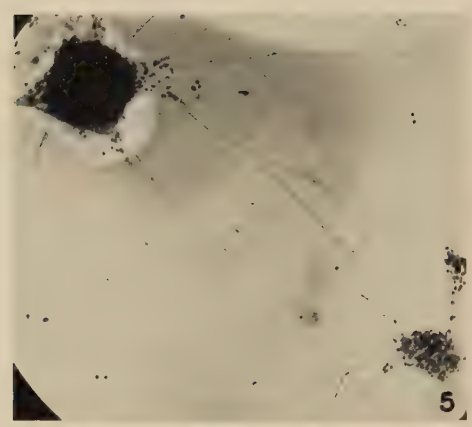
Fig. 1. Smear of a suspension of isolated 7-day spinal cord cells. Hematoxylin. 270 ×.
Fig. 2. Living 6-day spinal cord cells 2 hours after suspension was placed on clotted medium. Cells have aggregated into small clusters. 200 ×.
Fig. 3. Living 6-day spinal cord cells 1 day in culture. Clusters have enlarged. 200 ×. Same experiment as in Fig. 2, but different field.
Fig. 4. Living 7-day spinal cord 2 days in culture. Dark phase contrast. Nerve fibers have begun to bridge gaps between aggregated cells. 250 ×.

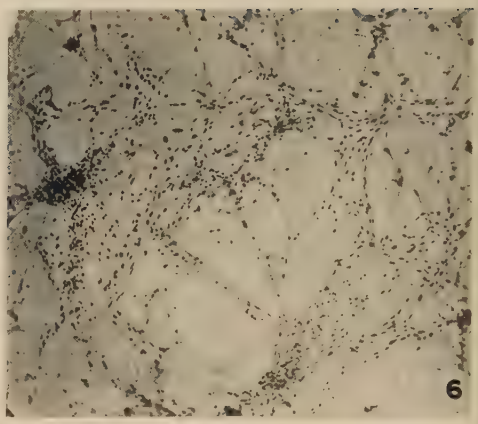
1

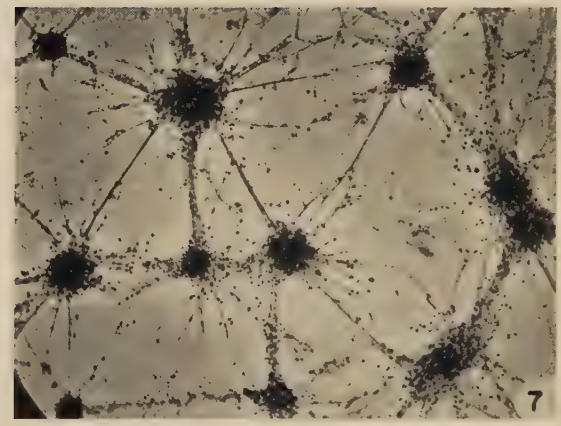

3


2

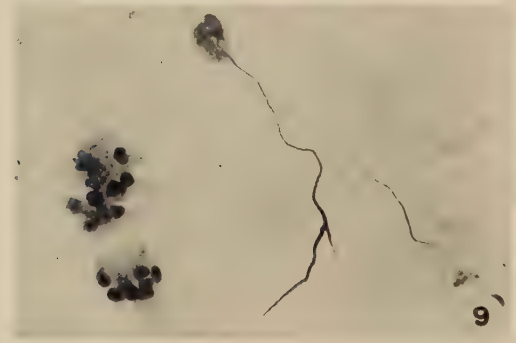

4


5


6


7

8


9

10

differences are presumably correlated with the position of the culture relative to the medium, whether in the interior, in a liquid pool, or on the surface.

The events following the initial stages of aggregation are essentially the same as those seen in regular tissue cultures of undissociated fragments of the nervous system. Fiber outgrowth continues for days and follows irregular courses, with merely a general prevalence of centrifugal directions. Neighboring clusters frequently become interconnected by bridges of cells or nerve fibers or both. This bridge effect ("two-center effect"; [7]) has been described and explained on previous occasions [6].

Secondary liquefaction in the vicinity of cell clusters later tends to crowd groups of nerve fibers into narrow strands spanning the pools of liquid, hence becoming aggregated into bundles [5].

Besides neurons, the cultures abounded in spindle cells and macrophages, evidently derived from the central glia. Since silver stains were used in most of the preparations, no systematic study of mitotic activity was possible.

The observations on mixed cultures, in which neuroblasts and other tissue cells had been intimately combined in artificial combinations, have not yet given conclusive results that could be reported at this time. The only observation that seems to be common to these experiments is that the neuroblasts tend to survive better and to sprout more extensively in the presence, than in the absence, of such admixtures. The effect may be a reciprocal one since the other cell types mixed with nerve cells likewise seemed to show more abundant growth than if they were raised in pure culture.

SUMMARY

Embryonic chick spinal cord in advanced stages of differentiation was dissociated into its constituent cells by trypsin digestion followed by mechanical separation. The dispersed cells, singly or in clusters, when seeded on or in blood plasma clots, developed typical nerve fibers up to several milli-

---

Fig. 5. Five-day spinal cord 3 days in culture. Extensive fiber outgrowth has formed a miniature plexus between two clumps of reaggregated cells. Protargol. 90 ×.

Fig. 6. Six-day spinal cord 4 days in culture. Sheet of cells has formed on the surface of the clot. Nerve fibers can be seen in the network of cells and spanning the liquefied areas of the clot. Protargol. 80 ×.

Fig. 7. Six-day spinal cord 4 days in culture. Same experiment as in Fig. 6. Clumps of cells have remained compact and well developed bundles of fibers have bridged the gaps between them. Protargol. 80 ×.

Fig. 8, 9 and 10. Isolated neurons with long fibers from a culture of 5-day spinal cord 3 days in culture. Protargol. 8. 210 ×. 9 and 10. 350 ×.

meters in length. This technique lends itself readily to studies of single neurons, as well as their reactions to controllable influences of the medium and of other cell types.

REFERENCES

1. Andres, G., *J. Exptl. Zool.* **122**, 507 (1953).
2. Moscona, A., *Exptl. Cell Research* **3**, 535 (1952).
3. Moscona, A. and Moscona, H., *J. Anat.* **86**, 287 (1952).
4. St. Amand, G. S. and Tipton, S. R., *Science* **119**, 93 (1954).
5. Weiss, P., *J. Exptl. Zool.* **100**, 353 1945).
6. —— In: Chemistry and Physiology of Growth, Princeton University Press, 135, 1949.
7. —— *Science* **115**, 293 (1952).
8. Weiss, P. and Andres, G., *ibid.* **111**, 456 (1950).
9. —— *J. Exptl. Zool.* **121**, 449 (1952).
10. Weiss, P. and Garber, B., *Proc. Natl. Acad. Sci.* **38**, 264 (1952).

Experimental Cell Research, 9, 49–67 (1955)

THE KINETICS OF MEMBRANE TRANSPORTS INVOLVING CHEMICAL REACTIONS

TH. ROSENBERG and W. WILBRANDT

Biochemical Department, Nordisk Insulinlaboratorium, Denmark, and Department of Pharmacology, University of Bern, Switzerland

Received September 22, 1954

There is an increasing number of observations on transports of solutes in biological systems showing qualitative and quantitative deviations from the laws of diffusion. Thus it has been found in various cases that the rate of transport is not proportional to the concentration difference, that competition between closely related molecules may occur, that chemical specificities of a surprising degree exist or that transports are affected by enzyme inhibitors. In many cases furthermore, transports have been shown to occur from lower to higher chemical potential.

Assumption of carrier mechanisms for these transports has frequently been made. Various aspects of such mechanisms have been discussed from the standpoint of thermodynamics by Rosenberg [9]. In the special case of glucose transport through the red cell membrane various observations along the lines mentioned [2, 4, 5, 10, 11, 12, 13, 14, 16, 17] led to the discussion of a carrier transport [2, 5, 7, 10, 11, 12, 17] and several treatments of the kinetics have been given [6, 8, 12, 15, 18].

In the present paper the kinetics of such transports are discussed in a more general way, by building up a system of enzymatic formation of membrane diffusible carrier substrate complexes from its components step by step and attempting to evaluate the contributions to the transport kinetics of the various elements in the complete system.

ASSUMPTIONS AND NOTATIONS USED

The assumptions on which the calculations will be based are the following:

The penetrating substrate S reacts with the carrier C in a first enzymatic reaction (enzyme E_1) at the membrane surface I to form the complex CS. The complex is capable to traverse the membrane by diffusion from surface I to surface II. At II it is split by a second enzymatic reaction (enzyme E_2), releasing S which is given off into the adjoining aqueous solution while C diffuses back from II to I.

For the sake of simplicity it will be assumed that S is soluble in water but insol-

uble in the membrane (implying that the membrane is impermeable for S as such), whereas C and CS are soluble in the membrane but not in water.

Such a system contains various elements which are not *a priori* dependent on each other: the necessity of a chemical reaction for the passage of the (otherwise impermeable) membrane, the formation of a complex with a carrier, the passage of this complex by diffusion and the enzymatic nature of the assumed chemical reactions.

Each of these elements contributes certain characteristics to the kinetics. These contributions may be evaluated if the system is gradually built from the elements mentioned. This will be done by treating the following systems along the same lines:

1. Penetration by a chemical reaction with a fixed membrane component C which combines with S at the side I of the membrane and releases S at the side II. Since no diffusion process in the strict sense of the word is involved this type will be called *reaction type.*

2. Penetration in a system of three successive stages: a chemical reaction at I, diffusion from I to II, and a second reaction at II. This combined type will be called *reaction-diffusion type.*

In the second case the reaction may either be a mere change of the substrate molecule (e.g. an isomeric transformation) to render it capable of membrane penetration, or the reaction may include a combination with a carrier C to form a complex CS capable of penetration. In both cases the assumption that the reactions are enzymatic will introduce special features.

The common element of the treatments will be the assumption of a steady state. This will be introduced by assuming the rates of transport to be equal at various cross sections of the system: at I and II in the first case, at I, II and within the membrane in the second.

The following symbols will be used:

v = rate of transport
S = substrate
C = carrier
CS = carrier substrate complex
T = transport form of the substrate
E = enzyme
(S) = substrate concentration
(C) = free carrier concentration
(CS) = concentration of the carrier substrate complex
$(C)_T$ = total carrier concentration $[=(C)+(CS)]$
(E) = total enzyme concentration
$a, b, \alpha, \beta, m_{\mathrm{I}}, m_{\mathrm{II}}, n_{\mathrm{I}}, n_{\mathrm{II}}$ = Velocity constants of the reactions according to equations (1)–(19). (1), (2), (8), (10), (12), (13), (16), (17), (25), (26), (27), (28).

K_r = equilibrium constant (dissociation constant) of the carrier substrate complex CS $\left(K_{r\,\mathrm{I}} = \frac{\alpha}{a},\ K_{r\,\mathrm{II}} = \frac{\beta}{b}\right)$

K_c = equilibrium constant (dissociation constant) of the carrier enzyme complexes (EC, ECS, SEC and $SECS$)

K_s = equilibrium constant (dissociation constant) of the substrate enzyme complexes (SE, SEC and $SECS$)

K_T = equilibrium constant (dissociation constant) of the complex ET

D' = $\frac{\text{diffusion coefficient} \times \text{surface area}}{\text{membrane thickness}}$ (assumed to be equal for the diffusing substances C and CS)

F = $\frac{(S)_{\mathrm{I}} - (S)_{\mathrm{II}}}{v}$ ("Transport resistance")

The indices I and II will refer to the surfaces I and II of the membrane. All activity coefficients are assumed to be = 1.

The Reaction Type

In this case C is not a "carrier" in the strict sense of the word since it is assumed to be a fixed membrane constituent, lacking mobility. This type is of course only a limiting case in which all diffusions are so rapid that they will never be rate determining. It is included in the present treatment partly because the model has been used [6] in order to explain experimental results on glucose transport in red cells and partly because of its formal similarity with other models discussed here. The same symbol C as for the true "carriers" in the following types is used for formal reasons.

The concentrations (C) and (CS) are constant throughout the membrane. Only the rates of the two reactions have to be considered which are assumed to be equal:

$$v = a(S)_{\mathrm{I}}(C) - \alpha(CS) \tag{1}$$

$$v = \beta(CS) - b(S)_{\mathrm{II}}(C). \tag{2}$$

Using the relations

$$(C)_T = (C) + (CS) \tag{3}$$

and

$$\frac{\alpha}{a} = \frac{\beta}{b} = K_r \tag{4}$$

and eliminating (C) and (CS), we arrive at

$$v = (C)_T \frac{(S)_{\mathrm{I}} - (S)_{\mathrm{II}}}{\frac{1}{a} + \frac{1}{b} + (S)_{\mathrm{I}} \cdot \frac{1}{\beta} + (S)_{\mathrm{II}} \cdot \frac{1}{\alpha}}. \tag{5}$$

The rate of transport thus is proportional to the total carrier concentration. The influence of the velocity constants and of the substrate concentrations becomes clearer if instead of v the "transport resistance" $F=\frac{(S)_I-(S)_{II}}{v}$ is used.

Then we obtain after some rearrangement:

$$F=\frac{1}{(C)_T}\left[\frac{1}{b}\left\{\frac{(S)_I}{Kr}+1\right\}+\frac{1}{a}\left\{\frac{(S)_{II}}{Kr}+1\right\}\right]. \tag{6}$$

The function F shows here (and also in other corresponding equations to be derived later) linear relationship to substrate-concentration. It is therefore particularly suited for kinetic characterisations. In the case of simple diffusion, F is a constant.

The kinetics appear to depend on the ratios $\frac{(S)}{K_r}$ and $\frac{a}{b}$. If both $(S)_I \ll K_r$ and $(S)_{II} \ll K_r$, i.e. if C is far from saturation the general form of the kinetics will be

$$v=\text{const.}\,[(S)_I-(S)_{II}] \tag{7}$$

which is identical with the kinetics of free diffusion and will be called type D. If on the other hand the substrate concentrations are so high that the carrier is near saturation (both $(S)_I \gg K_r$ and $(S)_{II} \gg K_r$), the general form of the kinetics will depend on the velocity constants, and, in the resulting types which are termed Z_1 and Z_2, v will be proportional to $\frac{(S)_I-(S)_{II}}{(S)_I}$ and $\frac{(S)_I-(S)_{II}}{(S)_{II}}$ respectively.

TABLE I

Forms of kinetics for the reaction type of transport.

Conditions for limiting velocity factors	Conditions for equilibrium (Saturation of the carrier)		
	$\frac{(S)_I}{Kr}<1$	$\frac{(S)_I}{Kr}>1$	$\frac{(S)_I}{Kr}>1$
	$\frac{(S)_{II}}{Kr}<1$	$\frac{(S)_{II}}{Kr}<1$	$\frac{(S)_{II}}{Kr}>1$
$\frac{1}{b}>\frac{1}{a}$	D	Z_1	Z_1
$\frac{1}{a}>\frac{1}{b}$	D	D	Z_2

For the singular case $a = b$ the combined type

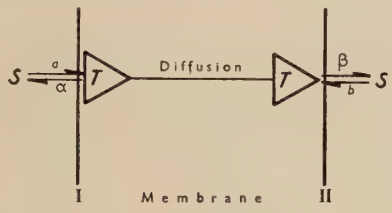
$$v = \text{const.} \frac{(S)_I + (S)_{II}}{(S)_I - (S)_{II}} \text{ appears which may be called } Z.$$

The Reaction-Diffusion Type

The reaction in such a transport system could be visualized in many different forms, e.g. $S \rightleftharpoons T$, $S + C \rightleftharpoons CS$, $2S + C \rightleftharpoons S_2C$, $S + 2C \rightleftharpoons SC_2$ etc. Of all these possible types only two shall be considered, namely the isomerisation and the reaction of one substrate molecule with one carrier molecule.

Non-enzymatic Transport Without Carrier

The reaction assumed will be the transformation of S into the isomer T at I, diffusion of T through the membrane and the back transformation at the surface II.



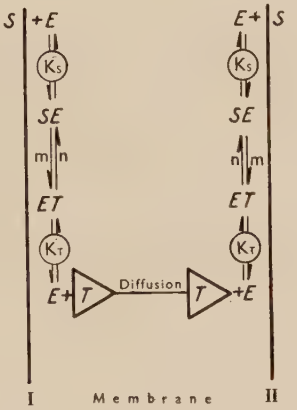


Fig. 1 (left). Scheme of the non-enzymatic isomerization type of reaction-diffusion transport.
Fig. 2 (right). Scheme of the enzymatic isomerization type of reaction-diffusion transport.

We may illustrate this model by a hypothetical transport of oxaloacetic acid from an aqueous layer I to an aqueous layer II through an ether layer. Since this substance appears to be completely enolized in ether whereas in aqueous solution only 3 per cent occur in the enol form, the rest in the keto form, the transport may be largely dependent on the isomerisation at the ether-water interfaces.

Treating this simplest case of the combined type by the same procedure as before, the following equations for the steady state are obtained:

$$v = \alpha(S)_I - \alpha(T)_I \tag{8}$$

$$v = [(T)_I - (T)_{II}]D' \tag{9}$$

$$v = \beta(T)_{II} - b(S)_{II}. \tag{10}$$

Eliminating $(T)_{\mathrm{I}}$ and $(T)_{\mathrm{II}}$ and assuming $\frac{\alpha}{a} = \frac{\beta}{b} = K_r$ we obtain

$$F = \frac{S_{\mathrm{I}} - S_{\mathrm{II}}}{v} = K_r \left(\frac{1}{\alpha} + \frac{1}{\beta} + \frac{1}{D'} \right). \tag{11}$$

The kinetics are of the type D irrespective of the ratio between substrate concentrations and K_r. This case is formally identical with that of diffusion through a lipoid membrane treated by Danielli [1], the chemical reaction velocity constants here replacing his velocity constants for the passage of the phase boundaries. If (as in Danielli's treatment) $\alpha = \beta$ we obtain for F the value $K_r \cdot \left(\frac{2}{\alpha} + \frac{1}{D'} \right)$ or for P, the "permeability constant" $\left(= \frac{1}{F} \right)$ the value $\frac{\alpha D'}{2D' + \alpha}$. This is formally identical with Danielli's value $\frac{ae}{nb + 2e}$, if $D' = \frac{e}{n}$ and $\alpha = a = b$.

Enzymatic transport without carrier.—A considerably higher degree of complexity is introduced by the assumption of an enzymatic nature of the reactions. In this case, the velocity constants a, b, α and β will have to be replaced by the following terms:

$$a = \frac{1}{K_S} \frac{m_{\mathrm{I}} E_{\mathrm{I}}}{1 + \frac{(S)_{\mathrm{I}}}{K_S} + \frac{(T)_{\mathrm{I}}}{K_T}}; \qquad \alpha = \frac{K_r}{K_S} \frac{n_{\mathrm{I}} E_{\mathrm{I}}}{1 + \frac{(S)_{\mathrm{I}}}{K_S} + \frac{(T)_{\mathrm{I}}}{K_T}} \tag{12 a) (12 b}$$

$$b = \frac{1}{K_S} \frac{m_{\mathrm{II}} E_{\mathrm{II}}}{1 + \frac{(S)_{\mathrm{II}}}{K_S} + \frac{(T)_{\mathrm{II}}}{K_T}}; \qquad \beta = \frac{K_r}{K_S} \frac{n_{\mathrm{II}} E_{\mathrm{II}}}{1 + \frac{(S)_{\mathrm{II}}}{K_S} + \frac{(T)_{\mathrm{II}}}{K_T}} \tag{13 a) (13 b}$$

(Assuming the same site of attachment at the enzyme molecule for both S and T, with the Michaelis constants K_S and K_T respectively.) The final equation then is:

$$F = K_r \left[\frac{1}{D'} \left\{ \left(1 + \frac{v}{n_{\mathrm{I}} E_{\mathrm{I}}} \right) \left(1 - \frac{v}{n_{\mathrm{II}} E_{\mathrm{II}}} \right) \right\} + \frac{K_T}{n_{\mathrm{I}} E_{\mathrm{I}}} \left\{ 1 + \frac{(S)_{\mathrm{I}}}{K_S} \left(1 - \frac{v}{n_{\mathrm{II}} E_{\mathrm{II}}} \right) + \frac{(S)_{\mathrm{II}}}{K_r K_T} \right\} \right.$$
$$\left. + \frac{K_T}{n_{\mathrm{II}} E_{\mathrm{II}}} \left\{ 1 + \frac{(S)_{\mathrm{I}}}{K_r K_T} + \frac{(S)_{\mathrm{II}}}{K_S} \left(1 + \frac{v}{n_{\mathrm{I}} E_{\mathrm{I}}} \right) \right\} \right] \tag{14}$$

which is rather remote from the simple form of equation (11). The equation is symmetrical in the sense that the rate of penetration will become zero if $(S)_{\mathrm{I}} = (S)_{\mathrm{II}}$. Furthermore it can be shown that for high values of S_{I}

a saturation value of v is reached (v_{max}). Thus, one of the consequences of the introduction of enzymes, as would be expected is the appearence of saturation phenomena.

In the case of different sites of attachment for S and T, the final equation is:

$$F = K_r \left[\frac{1}{D'} \left\{ \left(1 + \frac{v\left(1 + \frac{(S)_I}{K_S}\right)}{n_I E_I} \right) \left(1 - \frac{v\left(1 + \frac{(S)_{II}}{K_S}\right)}{n_{II} E_{II}} \right) \right\} \right.$$

$$\left. + \frac{K_T}{n_I E_I} \left\{ \left(1 + \frac{(S)_I}{K_S}\right)\left(1 + \frac{(S)_{II}}{K_r K_T}\right) \right\} + \frac{K_T}{n_{II} E_{II}} \left\{ \left(1 + \frac{(S)_I}{K_r K_T}\right)\left(1 + \frac{(S)_{II}}{K_S}\right) \right\} \right] \qquad (15)$$

The product $(S)_I(S)_{II}$ in the right side of (15) under certain conditions causes a type of kinetics termed E, which will be discussed later.

Non-enzymatic Transport With Carrier

If S reacts with a mobile carrier C to form a complex CS capable of membrane diffusion, the conditions differ from those of the reaction type in two points: the concentrations (C) and (CS) are not constant throughout the membrane and the rates of transport in three membrane elements instead of two have to be considered, the third concerning the diffusion in the membrane.

The equations replacing equations (1), (2) and (3) thus will be

$$v = a(S)_I(C)_I - \alpha(CS)_I \qquad (16)$$

$$v = \beta(CS)_{II} - b(S)_{II}(C)_{II} \qquad (17)$$

$$v = D'[(CS)_I - (CS)_{II}] \qquad (18)$$

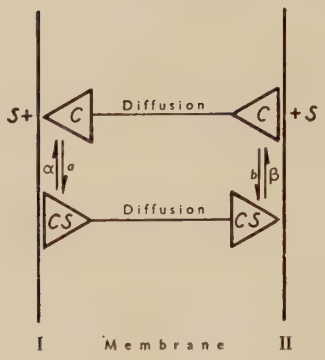
$$(CS)_I + (C)_I = (C)_T \qquad (19)$$

$$(CS)_{II} + (C)_{II} = (C)_T. \qquad (20)$$

Eliminating $(C)_I$, $(C)_{II}$, $(CS)_I$ and $(CS)_{II}$ from these five equations and substituting K_{rI} for $\frac{\alpha}{a}$ and K_{rII} for $\frac{\beta}{b}$, we obtain

$$\frac{K_{rII}(S)_I - K_{rI}(S)_{II}}{v} = \frac{1}{(C)_T}\left\{ \frac{(S)_I + K_{rI}}{b} + \frac{(S)_{II} + K_{rII}}{a} \right.$$

$$\left. + \frac{((S)_I + K_{rI})((S)_{II} + K_{rII})}{D'} \right\}. \qquad (21)$$



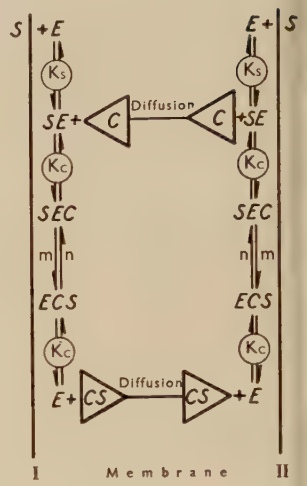


Fig. 3 (left). Scheme of the non-enzymatic carrier transport.
Fig. 4 (right). Scheme of the enzymatic carrier transport.

Assuming $K_{r\,\mathrm{I}} = K_{r\,\mathrm{II}} = K_r$ and rearranging, we obtain

$$F = \frac{(S)_\mathrm{I} - (S)_\mathrm{II}}{v} = \frac{1}{(C)_T}\left\{\frac{1}{b}\left[\frac{(S)_\mathrm{I}}{K_r} + 1\right] + \frac{1}{a}\left[\frac{(S)_\mathrm{II}}{K_r} + 1\right] + \frac{1}{D'}\left[\frac{(S)_\mathrm{I}}{K_r} + 1\right]\left[\frac{(S)_\mathrm{II}}{K_r} + 1\right]\right\} \tag{22}$$

which is identical with (6) in its first two terms. Since these terms contain reaction velocities as factors, they may be called reaction terms. Additionally equation (22) includes however a third term which under certain conditions may introduce a new type of kinetics. Since it contains $\frac{1}{D'}$ as factor it may be called diffusion term.

Again if both $(S)_\mathrm{I} \ll K_r$ and $(S)_\mathrm{II} \ll K_r$, irrespective of other conditions the kinetical type will be D (cf. the reaction type) and if both $(S)_\mathrm{I} \gg K_r$ and $(S)_\mathrm{II} \gg K_r$ and if $\frac{1}{D'} \ll \frac{1}{a}, \frac{1}{b}$, the kinetical types Z_1 or Z_2 may obtain.

If however $\frac{1}{D'} \gg \frac{1}{a}, \frac{1}{b}$ (the condition $(S) \gg K_r$ still holding), in other words if under saturation conditions the diffusion in the membrane is limiting, the aforementioned kinetical type E may appear, characterized by

$$v = \text{const.}\,\frac{(S)_\mathrm{I} - (S)_\mathrm{II}}{(S)_\mathrm{I}(S)_\mathrm{II}} \tag{23}$$

or

$$v = \text{const.} \left[\frac{1}{(S)_{II}} - \frac{1}{(S)_{I}} \right]. \tag{24}$$

The following table lists the types of kinetics to be expected under various conditions.

TABLE II

Forms of kinetics for the non-enzymatic carrier transport.

Conditions for limiting velocity factors	Conditions for equilibrium (Saturation of the carrier)		
	$\frac{S_I}{K_r} < 1$ $\frac{S_{II}}{K_r} < 1$	$\frac{S_I}{K_r} > 1$ $\frac{S_{II}}{K_r} < 1$	$\frac{S_I}{K_r} > 1$ $\frac{S_{II}}{K_r} > 1$
$\frac{1}{b} > \frac{1}{a}, \frac{1}{D'}$	D	Z_1	Z_1
$\frac{1}{a} > \frac{1}{b}, \frac{1}{D'}$	D	D	Z_2
$\frac{1}{D'} > \frac{1}{a}, \frac{1}{b}$	D	Z_1	E

For constant $(S)_{II}$ the types D and Z_2 are characterized by a linear relationship, between v and $(S)_I$, whereas Z_1 and E give saturation curves. Among the linear types the slope depends on $(S)_{II}$, for Z_2. Among the saturation types E is the only one in which the maximum rate depends on $(S)_{II}$.

Enzymatic Transport With Carrier

If the reactions are enzymatic, either two different enzymes or the same enzyme may catalyze the reaction. The kinetics in both cases will be of the same general type. Again terms containing enzyme concentrations $(E)_I$, $(E)_{II}$, velocity constants m and n of the reactions at the enzymes and Michaelis constants K_s and K_c will replace the velocity constants of equation (20):

$$a = (E)_I \frac{m_I}{[(S)_I + K_{sI}][(C)_T + K_{cI}]} \tag{25}$$

$$\alpha = (E)_I \frac{K_{sI} \cdot n_I}{[(S)_I + K_{sI}][(C)_T + K_{cI}]} \tag{26}$$

$$\beta = (E)_{II} \frac{K_{sII} \cdot n_{II}}{[(S)_{II} + K_{sII}][(C)_T + K_{cII}]} \tag{27}$$

$$b = (E)_{II} \frac{m_{II}}{[(S)_{II} + K_{sII}][(C)_T + K_{cII}]}. \tag{28}$$

In the derivation of these expressions it was assumed

a) that the enzyme molecules have two sites of attachment, one for S and one for either C or CS,

b) that the dissociation constant for one site is independent of whether the other is free or occupied,

c) that the affinities for C and CS have the same numerical value, and

d) that the splitting reaction $CS \rightarrow C + S$ can only occur if the site for S on the enzyme is free.

The equilibrium constants then will be

$$K_{rI} = \frac{\alpha}{a} = \frac{K_{sI} \cdot n_I}{m_I} \tag{29}$$

$$K_{rII} = \frac{\beta}{b} = \frac{K_{sII} \cdot n_{II}}{m_{II}} \tag{30}$$

Assuming $K_{rI} = K_{rII} = K_r$, furthermore $K_{cI} = K_{cII} = K_c$ and $K_{sI} = K_{sII} = K_s$, the final relation arrived at for this type of transport is

$$F = \frac{(S)_I - (S)_{II}}{v} = K_r \left[\left(1 + \frac{(S)_I}{K_S}\right)\left(1 + \frac{(S)_{II}}{K_r}\right)\left(1 + \frac{K_c}{C_T}\right)\frac{1}{E_I n_I} \right.$$
$$\left. + \left(1 + \frac{(S)_I}{K_r}\right)\left(1 + \frac{(S)_{II}}{K_S}\right)\left(1 + \frac{K_c}{C_T}\right)\frac{1}{E_{II} n_{II}} + \left(1 + \frac{(S)_I}{K_r}\right)\left(1 + \frac{(S)_{II}}{K_r}\right)\frac{1}{C_T D'} \right] \tag{31}$$

The right side of this equation again consists of two reaction terms and one diffusion term, each of which may become limiting. The diffusion term is the same as in equation (22). In the reaction terms however, the expressions $\left(1 + \frac{S}{K_S}\right)$ appear, which introduce a new possibility: the type E may now arise not only if the diffusion term is limiting and the carrier saturated (both $(S)_I \gg K_r$ and $(S)_{II} \gg K_r$) but also if one of the reaction terms is limiting and if at the same time at least one enzyme is saturated. Table III lists the conditions under which the various types of kinetics emerge.

In this treatment the original Michaelis concept of equilibrium between enzyme and substrates has been maintained. Considerable doubt, however,

has been thrown by several authors (e.g. [3]) on the general validity of such an assumption. Some modifications introduced in the above equations, when the conditions of equilibrium are abandoned, will be discussed in a following paper.

TABLE III

Forms of kinetics for the enzymatic carrier transport.

Conditions for limiting rate factors	Conditions for saturation of the enzymes $\frac{(S)_I}{K_S}$	$\frac{(S)_{II}}{K_S}$	Conditions for saturation of the carrier $(S)_I < K_r$ $(S)_{II} < K_r$	$(S)_I < K_r$ $(S)_{II} > K_r$	$(S)_I > K_r$ $(S)_{II} > K_r$
$\frac{1}{(E)_I m_I} > \frac{1}{(E)_{II} m_{II}}, \frac{1}{M_c}$	<1	<1	D	D	Z_2
	>1	<1	Z_1	Z_1	E
	>1	>1	Z_1	Z_1	E
$\frac{1}{(E)_{II} m_{II}} > \frac{1}{(E)_I m_I}, \frac{1}{M_c}$	<1	<1	D	Z_1	Z_1
	>1	<1	D	Z_1	Z_1
	>1	>1	Z_2	E	E
$\frac{1}{M} > \frac{1}{(E)_I m_I}, \frac{1}{(E)_{II} m_{II}}$	<1	1	D	Z_1	E
	>1	1			
	>1	1			

The Kinetical Asymmetry

One further aspect of the transport system has to be discussed: the symmetry relationships with regard to the two sides of the membrane. The system may be symmetrical or asymmetrical in two respects: as regards either the reaction velocities or the equilibria, in other words either with respect to the kinetics or with respect to the energetics.

Energetically up to this point the assumption of symmetry was implicitly maintained. Asymmetry which implies accumulation will be treated in the following chapter.

Kinetically however an analogous assumption was not generally introduced in the treatment. In the general case of the reaction-diffusion type, e.g. in equation (22) it was not assumed that $a = b$.

The kinetical asymmetry thus introduced implies that the rate of transport under comparable conditions (exchanged substrate concentrations) may depend on its direction. This may e.g. be seen from the fact that exchanging $(S)_I$ and $(S)_{II}$ in equation (22) results in a change in the transport rate (v), if $a \neq b$. Fig. 5 shows the effect for arbitrarily chosen numerical values. In

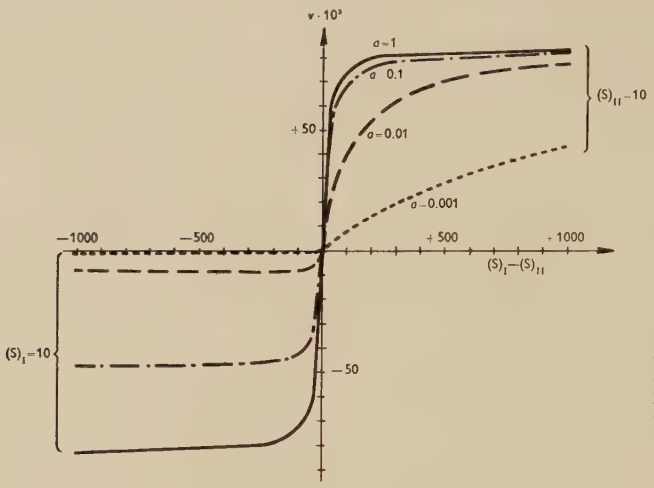


Fig. 5 Kinetical asymmetry of the non-enzymatic carrier transport. Ordinate: Rate of transport. Abscissa: Concentration difference across the membrane.

order to have comparable points (with equal gradients) symmetrically arranged, in this figure v has been plotted against the concentration difference $[(S)_I - (S)_{II}]$ for constant $(S)_I$ or $(S)_{II}$, respectively (according to the direction of the transport). For the chosen values ($K_r = 1$, $D' = 1$, $C_T = 1$, $b = 1$, $(S)_I$ or $(S)_{II} = 10$) the maximum rates will be

$$v_{max} = \frac{1}{11 + \frac{1}{b}} = \frac{1}{12} \tag{32}$$

in the direction I→II, and

$$v_{max} = \frac{1}{11 + \frac{1}{a}} \quad \text{in the other.} \tag{33}$$

Thus varying only one of the reaction velocity constants will affect the maximum rate of transport only in one direction. The general conditions for the occurrence of this type of asymmetry will be discussed later.

Energetical Asymmetry and Accumulation

If in equation (21) K_{rI} and K_{rII} are not true equilibrium constants, as assumed up to this point, but contain other activitities due to the participation of other components in the reactions considered, accumulation may result under certain conditions.

This may be seen from (21) where $v = 0$ for $K_{rII}(S)_I = K_{rI}(S)_{II}$. Consequently, the accumulation ratio in equilibrium will be:

$$\frac{(S)_{II}}{(S)_{I}} = \frac{K_{r\,II}}{K_{r\,I}} \tag{34}$$

A possible form of such a participation of additional reactants would appear to be the following. Suppose that the substrate-carrier reaction at the surface I is coupled with a reaction $U \rightarrow V$, and the splitting reaction at the surface II with a reaction $Y \rightarrow Z$ (where each of the symbols U, V, Y, and Z may represent one or several components), the overall reaction then will be:

$$C_{I} + S_{I} + U_{I} \underset{\alpha'}{\overset{a'}{\rightleftharpoons}} CS_{I} + V_{I} \tag{35}$$

and

$$CS_{II} + Y_{II} \underset{b'}{\overset{\beta'}{\rightleftharpoons}} C_{II} + S_{II} + Z_{II}. \tag{36}$$

The equations for the steady state at I and II become

$$v = a'(S)_{I}(C)_{I}(U)_{I} - \alpha'(CS)_{I}(V)_{I} \tag{37}$$

and

$$v = \beta'(CS)_{II}(Y)_{II} - b'(S)_{II}(C)_{II}(Z)_{II}. \tag{38}$$

It can readily be seen that equations (37) and (38) emerge from (16) and (17), if the velocity constants of the reactions are replaced by the following terms:

$$a \quad \text{by} \quad a'(U)_{I}$$

$$b \quad \text{by} \quad b'(Z)_{II}$$

$$\alpha \quad \text{by} \quad \alpha'(V)_{I}$$

$$\beta \quad \text{by} \quad \beta'(Y)_{II}$$

$$K_{r\,I} \quad \text{by} \quad \frac{\alpha'}{a'}\frac{(V)_{I}}{(U)_{I}}$$

$$\text{and} \quad K_{r\,II} \quad \text{by} \quad \frac{\beta'}{b'}\frac{(Y)_{II}}{(Z)_{II}}.$$

Substituting these expressions in equation (34) we obtain for the accumulation ratio

$$\frac{(S)_{II}}{(S)_{I}} = \frac{\beta'}{b'}\frac{a'}{\alpha'}\frac{(U)_{I}(Y)_{II}}{(V)_{I}(Z)_{II}}. \tag{39}$$

If one mole of the substrate is transported across the membrane, one mole of U_{I} and Y_{II} each will be transformed to V_{I} and Z_{II}. The minimum work of transport at equilibrium thus will be equal to the difference of the free energies of the overall reaction and of the reaction

$$U_{\mathrm{I}} + Y_{\mathrm{II}} \rightarrow V_{\mathrm{I}} + Z_{\mathrm{II}} \tag{40}$$

or

$$RT \ln \frac{(S)_{\mathrm{II}}}{(S)_{\mathrm{I}}} = RT \ln \frac{\beta' a'}{b' \alpha'} - RT \ln \frac{(V)_{\mathrm{I}}(Z)_{\mathrm{II}}}{(U)_{\mathrm{I}}(Y)_{\mathrm{II}}}. \tag{41}$$

It appears to be a general feature of active transport mechanisms that some of the reactants of the energy supplying reaction must be spatially separated (cf. [9]).

DISCUSSION

The present kinetical analysis of transports involving chemical reactions has been undertaken in order to make possible or to facilitate the recognition and classification of biological transports which appear to depend on chemical reactions.

The kinetics derived are based on complex assumptions and contain a number of variables. It appeared useful therefore to analyse the individual contributions of the various partial assumptions as far as possible. The result of this analysis will now be briefly discussed.

For the purpose of the analysis of experimental data the chief interest of the equations derived lies in the relationship between substrate concentrations and rate of transport, because these are the only values which in general will be known.

This relationship in some cases was shown not to differ from that of simple diffusion (kinetical type D). Thus the experimental finding that the kinetics of a transport obey the laws of diffusion does by no means justify the conclusion that the transport is merely a passive diffusion process not involving chemical reactions.

Special features may however appear either in the form of "saturation kinetics" (E or Z_1), i.e. kinetics with constant rate of transport for high substrate concentrations, or in the form of the Z_2 kinetics, in which the rate is a linear function of $(S)_{\mathrm{I}}$ but with a slope depending on $(S)_{\mathrm{II}}$ in a manner not shared by simple diffusion. Of these in general the saturation type will be more striking and more easily recognized since in many cases $(S)_{\mathrm{II}}$ will be unknown or less accurately known than $(S)_{\mathrm{I}}$.

For the further discussion it is felt useful to designate a transport type with a potential constant maximal rate as a "saturation type" and to call it saturated, if the rate has actually reached the maximum.

Two main questions then arise:

1. What is the prerequisite for saturation types of transports?
2. Under which conditions will transports in such systems actually be saturated?

The participation of a chemical reaction alone does not necessarily lead to unusual kinetic features, as shown by the example of non-enzymatic isomerisation which under all conditions yields *D*-kinetics. Saturation types

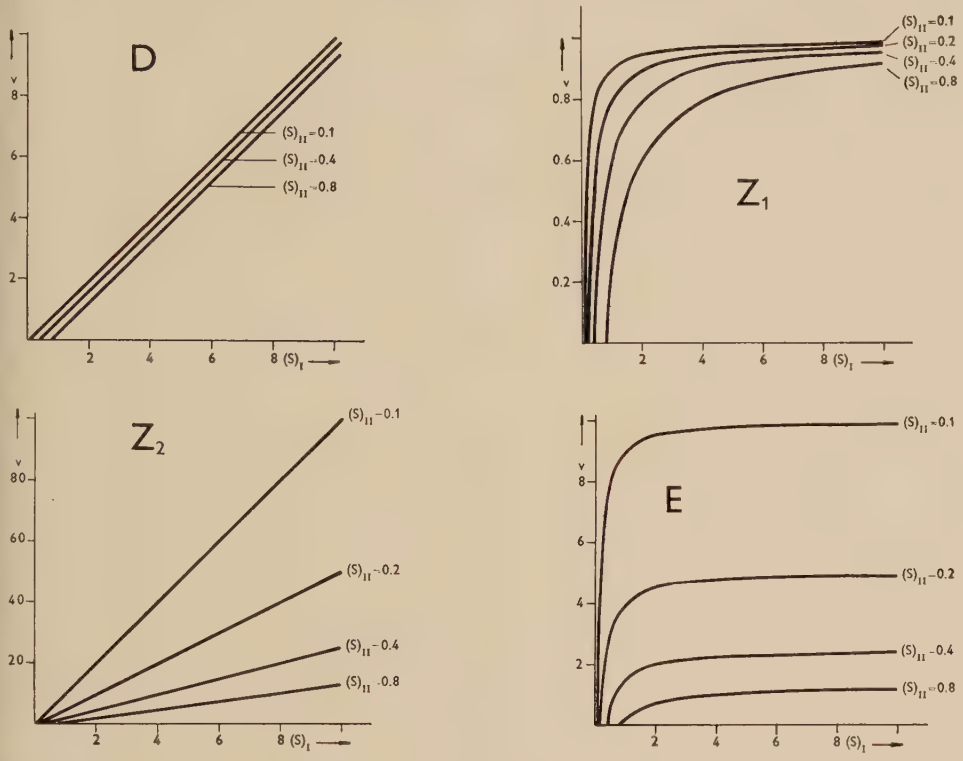


Fig. 6. Types of transport kinetics, which may emerge under different conditions as to limiting factors.

only appear if either carriers or enzymes (or both) are involved. This is of course due to the fact that these components, being present in a limited amount, may become saturated with the penetrating substrate *S*.

In fact, as can readily be seen from Tables I, II and III, actual saturation of the transport only occurs if at least one of the four components (E_I E_{II} C_I C_{II}) is saturated. Otherwise for all transport types considered, the kinetical type is always *D*.

There are, however, a number of facts which show that the relationship

between the saturation of these components and the "saturation" of the transport is by no means quite simple.

On the one hand there are certain conditions under which, in spite of saturation of the enzyme or of the carrier on one side of the membrane, the kinetics type is D, or in spite of saturation of enzyme or carrier on both sides the type is Z_2 which likewise is not a saturation type. Examples are seen in Tables II and III.

Furthermore, although the types Z_1 and E are both saturation types, they differ in a way that one might characterize by calling E a saturation type of higher order than Z_1.

Such a distinction may be justified by the fact that the equations relating v and $(S)_I$ for constant values of $G=(S)_I-(S)_{II}$ actually are of zero order for D, of first order for Z_1 and Z_2 and of second order for E. The reciprocal of the rate $\frac{1}{v}$ under these conditions will be proportional to

$$\frac{1}{G},\ \frac{(S)_I}{G},\ 1-\frac{(S)_I}{G} \text{ and } (S)_I-\frac{(S)_I^2}{G} \text{ respectively.}$$

Thus, the further question arises as to the special conditions favouring the appearance of such a saturation type of higher order.

It can immediately be seen from Tables I—III that E only arises if at least two sites are saturated. But although this condition appears necessary, it is obviously not sufficient, since a number of conditions in the enzymatic types of transport (Tables II and III) lead to type Z_1 or even Z_2, although two sites are saturated. For the enzymatic carrier transport, Fig. 7 shows the particular combinations of saturation at two sites which lead to E kinetics.

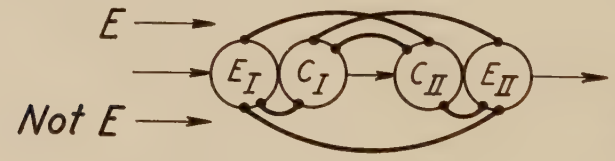


Fig. 7. Pairs of saturated sites, which in the enzymatic carrier transport will or will not lead to the kinetical type E.

One point of interest emerges from the scheme, namely that in all the combinations leading to E the two saturated sites are on different sides of the membrane. But again this condition is not sufficient, since saturation of both enzymes without carrier saturation does not yield E.

A useful point of view leading to a more general statement appears to be the following. In all reaction types, either a mere change of the molecule or a combination with a carrier transforms the substrate into a trans-

port form which may be called T. The difference $(T)_I - (T)_{II}$ then will always be proportional to the rate v, independent of the transport type and conditions. The function relating v, $(S)_I$ and $(S)_{II}$, will therefore depend on the relationships between $(T)_I$ and $(S)_I$ and between $(T)_{II}$ and $(S)_{II}$. It can be shown (the derivation will not be given here for lack of space) $(T) = \frac{A + B(S)}{(S)}$ (A and B being functions of v).

Thus saturation values of both $(T)_I$ and $(T)_{II}$ appear to be the condition for the occurrence of the "second order saturation" type E.

The dependence of the kinetical type on the degree of saturation at the sites E_I, E_{II}, C_I, C_{II}, as it was just discussed, can be represented in several ways. One of them is the form of equation (31). For the practical purpose of analysis of experimental data this is certainly the most useful form since it shows the influence of the substrate concentrations, and the conditions for the appearance of the various special types of kinetics can readily be seen. A different form however can be derived from equation (31) which is somewhat simpler and shows the effect of saturation from a different angle. It is obtained by introducing (E_{ff}) and (C_f), the concentrations of free enzyme (bound neither to S nor to C or CS), and of free carrier (not bound to S). The equation then takes the form

$$F = K_c K_r \left[\frac{1}{n_I} \frac{1}{(E_{ff})_I} \frac{1}{(C_f)_{II}} + \frac{1}{n_{II}} \frac{1}{(E_{ff})_{II}} \frac{1}{(C_f)_I} + \frac{(C_T)}{D' K_c} \frac{1}{(C_f)_I} \frac{1}{(C_f)_{II}} \right]. \qquad (42)$$

It appears from the equation, that the additive terms of the "transport resistance" F can each be regarded as the reciprocal product of two free concentrations (E_{ff}) or (C_f). Since in this form both the total concentrations $(E)_I$ $(E)_{II}$ (C_T) and the Michaelis-Menten terms $\left(1 + \frac{(S)}{K_s}\right)$, $\left(1 + \frac{(S)}{Kr}\right)$ have cancelled out, (C_f) and (E_{ff}) appear to be of primary importance for the resistance.

It was shown that an asymmetry with respect to the rate of transport may arise under certain conditions which shall now be discussed. The total transport contains elements of diffusion and of chemical reactions. Whereas the conditions for diffusion of the transport form are symmetrical with respect to the two sides of the membrane, those for the chemical reactions may be different, due to different concentrations of other reactants or catalysts, different surface tensions or the like. This is in accordance with the fact that the values of F or v in equations (6), (14), (15), (22) and (31) will not be affected by exchanging $(S)_I$ and $(S)_{II}$ in the diffusion terms, while such

an exchange in the reaction terms may change the numerical value of F or v. This means, that an experimental finding of such an asymmetry may be taken as an indication of chemical reactions being involved in the transport concerned.

On the other hand asymmetry is not a necessary consequence of the participation of chemical reactions. If the kinetical type is E or D, differences of the reaction rates at the two sides will not lead to different transport rates in the two directions. This type of asymmetry is restricted to types Z_1, Z_2, and mixed types.

Coupling of the transport to exergonic reactions of spatially separated components may result in accumulation. The equilibrium relationships of such a system have been shown to be relatively simple. The kinetics may, however, be rather involved unless the concentrations of the components participating in the energy yielding reactions are constant. In this case the equations derived above for the reaction diffusion type remain valid.

SUMMARY

1. The kinetics of various transport systems across cell membranes involving chemical reactions of the transported substrate in the membrane (with and without carrier molecules) have been worked out for the steady state.

2. It is shown that of four kinetical types which were termed D, Z_1, Z_2 and E anyone may represent the kinetical type of the transport either generally or under particular conditions as regards limiting factors.

3. In these types the dependence of the rate of transport v on the substrate concentrations $(S)_{\text{I}}$ and $(S)_{\text{II}}$ takes the following forms (K_D, K_{Z_1}, K_{Z_2} and K_E being constants):

Type D $$v = K_D[(S)_{\text{I}} - (S)_{\text{II}}]$$

Type Z_1 $$v = K_{Z_1}\frac{(S)_{\text{I}} - (S)_{\text{II}}}{(S)_{\text{I}}}$$

Type Z_2 $$v = K_{Z_2}\frac{(S)_{\text{I}} - (S)_{\text{II}}}{(S)_{\text{II}}}$$

Type E $$v = K_E\frac{(S)_{\text{I}} - (S)_{\text{II}}}{(S)_{\text{I}}(S)_{\text{II}}} = K_E\left[\frac{1}{(S)_{\text{II}}} - \frac{1}{(S)_{\text{I}}}\right].$$

D is the type known from diffusion transports. In the types Z_1 and E, the rate v becomes constant for high substrate concentration. E is characterized

by a dependence of the saturation level on the value of $(S)_{II}$ $\left(\frac{1}{v_{max}}\right.$ proportional to $\left.(S)_{II}\right)$. Furthermore various mixed types may occur.

4. It is shown that

a) the mere participation of a chemical reaction does not necessarily introduce new types of kinetics.

b) with low substrate concentrations all systems follow the type *D*.

c) the other types may result from the participation of carrier or enzyme molecules if at least one of them is saturated.

d) the type *E*, which may be considered as saturation type of higher order, is bound to the condition that at least two sites (on different surfaces of the membrane) are saturated with *S*.

5. Kinetical asymmetry, i.e. different rates of transport in the two opposite directions, when the substrate concentrations are exchanged, may under certain restricted conditions result from differences in the corresponding velocity constants or enzyme concentrations.

6. The mechanism of coupling to energy-supplying reactions, resulting in accumulation, has been considered from analogous points of view.

REFERENCES

1. Davson, H. and Danielli, J. F., The permeability of natural membranes. Cambridge University Press 1943.
2. Guensberg, E., Inaugural-Dissertation Bern, 1947.
3. Kauman, S., Neurath, H., and Schwert, G. W., *J. Biol. Chem.* **177**, 793 (1949).
4. Le Fèvre, P. G., *Biol. Bull.* **93**, 224 (1947).
5. —— *J. Gen. Physiol.* **31**, 505 (1948).
6. —— *Symposia Soc. exptl. Biol.* **VIII** (1954) (in press).
7. Le Fèvre, P. G. and Davies, R. I., *J. gen. Physiol.* **34**, 515 (1951).
8. Le Fèvre, P. G. and Le Fèvre, M. E., *ibid.* **35**, 891 (1952).
9. Rosenberg, Th., *Acta Chem Scand.* 2, 14 (1948).
10. Rosenberg, Th. and Wilbrandt, W., *Internatl. Rev. Cytology* **1**, 65 (1952).
11. Widdas, W. F., *J. Physiol.* **115**, 36 P. (1951).
12. —— *J. Physiol.* **118**, 23 (1952).
13. —— Thesis, University of London, 1953.
14. Wilbrandt, W., *Helv. Physiol. Acta* **5**, C 64 (1947).
15. —— *Symposia Soc. exptl. Biol.* **VIII** (1954) (in press).
16. Wilbrandt, W., Guensberg, E., and Lauener, H., *Helv. Physiol. Acta*, **5**, C 20 (1947).
17. Wilbrandt, W. and Rosenberg, Th., *ibid.* **8**, C 82 (1950).
18. —— *ibid.* **9**, C 86 (1951).

THE DISTRIBUTION OF THE DRY WEIGHT IN SQUAMOUS EPITHELIUM DURING CARCINOGENESIS STUDIED BY MEANS OF QUANTITATIVE ROENTGEN ABSORPTION SPECTROPHOTOMETRY

B. LINDSTRÖM and G. MOBERGER[1]

The Institute for Cell Research, Medical Nobel Institute, and the Institute of Radiopathology, Karolinska Institutet, Stockholm, Sweden

Received September 25, 1954

From chemical investigations differences in the quantitative chemical composition between malignant and homologous non-malignant tissues have been revealed [27]. Extensive studies on mouse epidermis during carcinogenesis, for example, have shown an increase in water content and a decrease in protein nitrogen and mineral content per unit volume of tissue during the formation of the malignant tumors [3, 8]. However, great difficulties are involved in interpreting the results obtained from chemical analyses in terms changes in the individual cells or even in the different layers of the epidermal tissues [8]. Cytochemical methods utilizing the absorption of radiant energy have made possible the study of the quantitative distribution of certain compounds in cellular structures *in situ*. These techniques have thus opened new pathways to the elucidation of chemical changes accompanying carcinogenesis at the cellular level.

By means of ultraviolet absorption microspectrophotometry Caspersson and Santesson [7] found great variations in the absorption capacity of cells in different parts of a number of epithelial tumors. The cells in the outer layers of solid cancer cords generally had a high absorption at about 260 mμ, indicating high concentrations of nucleic acids and proteins (A-cells). In the inner layers of the cancer cords the cells were distinguished by lower absorption and hence lower concentration of these substances (B-cells). These differences in the ultraviolet absorption of cells in different layers of cancer cords were ascribed partly to nutritional factors. When compared with the basal layer of normal epidermis, the outer cell layers of the cords in infiltrating portions of epidermoid carcinomas generally had a higher ultraviolet absorption [17].

[1] This investigation has been supported by a grant from the Swedish Cancer Society which we greatfully acknowledge.

In biological material, variations in the dry weight of different structures influence the absorption values obtained by means of microspectrophotometry in different wave length ranges. Therefore, the determination of dry weight in thin sections of tissue is a valuable aid to the quantification of results obtained with other cytochemical methods. In 1949–50 Engström and Lindström [12] introduced a method utilizing the absorption of soft continuous roentgen rays for the quantitative determination of the dry weight of minute tissue structures. Historadiographic studies on normal, hyperplastic and cancerous epidermis revealed great variations in the distribution of the dry weight of neoplastic tissues [19]. In contrast, the normal epidermis was distinguished by a fairly homogeneous distribution of the dry weight in the different layers. By means of quantitative roentgen absorption spectrophotometry the mean dry weights per unit area could be determined in the vaginal squamous epithelium and in derived squamous cell carcinomas. The experimental error of the method was less than $\pm$ 10 per cent [14, 16].

The present paper is a description of changes in the distribution of the dry weight in different types of squamous epithelium during carcinogenesis as studied by quantitative spectrophotometry using the absorption of extremely soft, continuous roentgen rays.

MATERIAL

The squamous epithelium of the vaginal mucosa and the epidermis of the mouse were selected for the roentgen absorption analyses. Twenty-five C_3H-mice aged four weeks and with an average weight of 20 grams were used. One coco nut fat pellet, containing 10 mg of methylcholanthrene, was inserted into the vaginal cavity of each animal and the *introitus vaginae* was then closed with catgut sutures. The mice were sacrificed at various intervals, and in a few animals the treatment was repeated two months after the first application of the carcinogen. All stages from precancerous hyperplasia via papillomatous, non-infiltrating tumors to fully developed squamous cell carcinomas were obtained. By scratching the vaginal mucosa with a sharp forceps, non-tumorous regenerating hyperplasia was achieved on the edges of the healing wounds in a few mice. All specimens were taken at the same level, about 0.5 cm from the *orificium vaginae*.

Methylcholanthrene in a 0.6 per cent solution in acetone was applied to the skin of twenty C_3H-mice by brushing the backs of the mice twice a week. Within four to five months papillomatous tumors appeared on the painted areas, and a few epidermoid carcinomas developed six months after the initial application of the carcinogen.

The tissue pieces were frozen-dried with the equipment and technique described previously [20]. Paraffin-embedding of the frozen-dried specimens was carried out *in vacuo*. Some tissue pieces were also fixed in 10 per cent neutral formalin for 24 hours and embedded in paraffin in the usual way. Several formalin-fixed human biopsy specimens, representing normal epidermis and various stages in the development of epidermoid carcinoma, were also analysed.

METHODS

The technique of quantitative roentgen absorption spectrophotometry was applied to the determination of dry weight [12, 14]. A complete account of the general theory, the apparatus and the detailed procedure for roentgen absorption analysis will be published elsewhere [15]. The specimen (a microtome section 5–7μ thick) was mounted on a zapon varnish foil over a slit on a metal holder together with a reference system, consisting of a step wedge of parlodion foils. The sections were deparaffinized in chloroform for ten minutes. With continuous soft roentgen rays, generated at 1500 volts and filtered through 9μ Al, a contact picture of the specimen and the reference system was registered on a Kodak Maximum Resolution Plate, which was in close contact with the specimen [14]. After development, the densities in the absorption picture of the sample and of the reference wedge were compared by means of direct photometry in the enlarged image of the microradiogram. A secondary magnification of about 400 times was possible without disturbing grain in the image. The fine-grained emulsion had a rather low gamma-value, but the extremely soft roentgen rays used were strongly absorbed by the biological objects. A high contrast was therefore obtained in the microradiograms.

In the epithelial tissues analysed in the present investigation most of the organic material consisted of proteins. Most of the lipids were dissolved out during the deparaffinizing procedure. As the concentration of other organic components is much lower than that of proteins, the dry weights per unit area obtained from the roentgen absorption determinations were an approximate measure of the protein concentration in different structures [12, 19].

The dry weights per unit volume can be obtained if the thickness of the sections is accurately known. However, determinations of the thickness of histological sections are subject to relatively large errors. In this study the ratios between the dry weights per unit volume of different structures were obtained without determining the absolute thickness of the sections. This was achieved by embedding the two tissues to be compared in the same block of paraffin and cutting them simultaneously. In order to avoid a wedge-effect during the cutting of the sections, the paraffin-blocks were alternately cut from two directions [16]. The ratio of the weights per unit area in these absorbing layers of equal thickness is thus also a measure of the ratio of their weights per unit volume. Provided that the elementary composition of the tissues is approximately the same, the systematic error of the method is negligible when ratios of dry weights are determined.

RESULTS

Experimental material.—The squamous epithelium of the vaginal mucosa of the mouse comprised between five and ten cell layers. No differences in the dry weight were found in the different cellular layers of the *normal vaginal epithelium* (Fig. 1 a).

Mice in the proestrous and in the diestrous stage of the estrous cycle were selected by means of cytological examination of vaginal smears made

by the lavage method. No significant difference in the dry weight per unit volume of tissue was found between the frozen-dried squamous epithelium in the two stages (ratio proestrous/diestrous 0.98).

A homogeneous distribution of the dry weight was also found in the different layers of the frozen-dried, slightly *hyperplastic regenerating squamous epithelium* (Fig. 1 b). When compared with the normal epithelium the mean dry weight per unit volume of tissue in the hyperplastic epithelium was significantly lower. The mean ratio between the two was found to be 0.81 with a standard error of the mean of ± 3 per cent, based upon 21 individual ratios, each derived from 40 photometric readings.

In the frozen-dried *precancerous hyperplastic epithelium* the dry weight was fairly homogeneously distributed in the different layers (Fig. 1 c). The mean ratio between the mean dry weights per unit volume of the precancerous and the normal epithelium was 0.73 with a standard error of the mean of ± 5 per cent. This decrease in the dry weight of the precancerous epithelium when compared with the normal was highly significant ($P < 0.001$).

A thin layer of keratinized material at the surface of the regenerating and the precancerous, hyperplastic epithelium had a rather high, though somewhat varying dry weight (Fig. 1 b and c).

In the frozen-dried *squamous cell carcinomas* a moderate variation of the dry weight was found in the different layers of the cancer cords (Fig. 2 a). No significant difference was found between the dry weights per unit volume of the outer cell layers of seventeen cancer cords and the basal layer of the normal epithelium (mean ratio 0.94 with a standard error of the mean of ± 7 per cent, ca. 400 photometric readings). The inner layers of the cancer cords had a lower dry weight than the spinous layer of normal epithelium (mean ratio 0.66 with a standard error of the mean of ± 5 per cent, ca. 300 photometric readings). Keratinized structures in the carcinomas had a considerably higher dry weight and were excluded from the measurements.

In the formalin-fixed normal and hyperplastic vaginal squamous epithelium the dry weight was generally somewhat higher than in the frozen-dried material. The most pronounced differences in the distribution of the dry weight were, however, found in formalin-fixed squamous cell carcinomas (Fig. 2 b). In this material a higher dry weight per unit volume was generally found as compared with that of corresponding structures in frozen-dried specimens. In the formalin-fixed material the mean ratio of the dry weight between the outer cell layers of 24 individual cancer cords and the basal layer of the normal epithelium was 1.50 with a standard error of the mean of ± 4 per cent. In the same material the corresponding mean ratio between

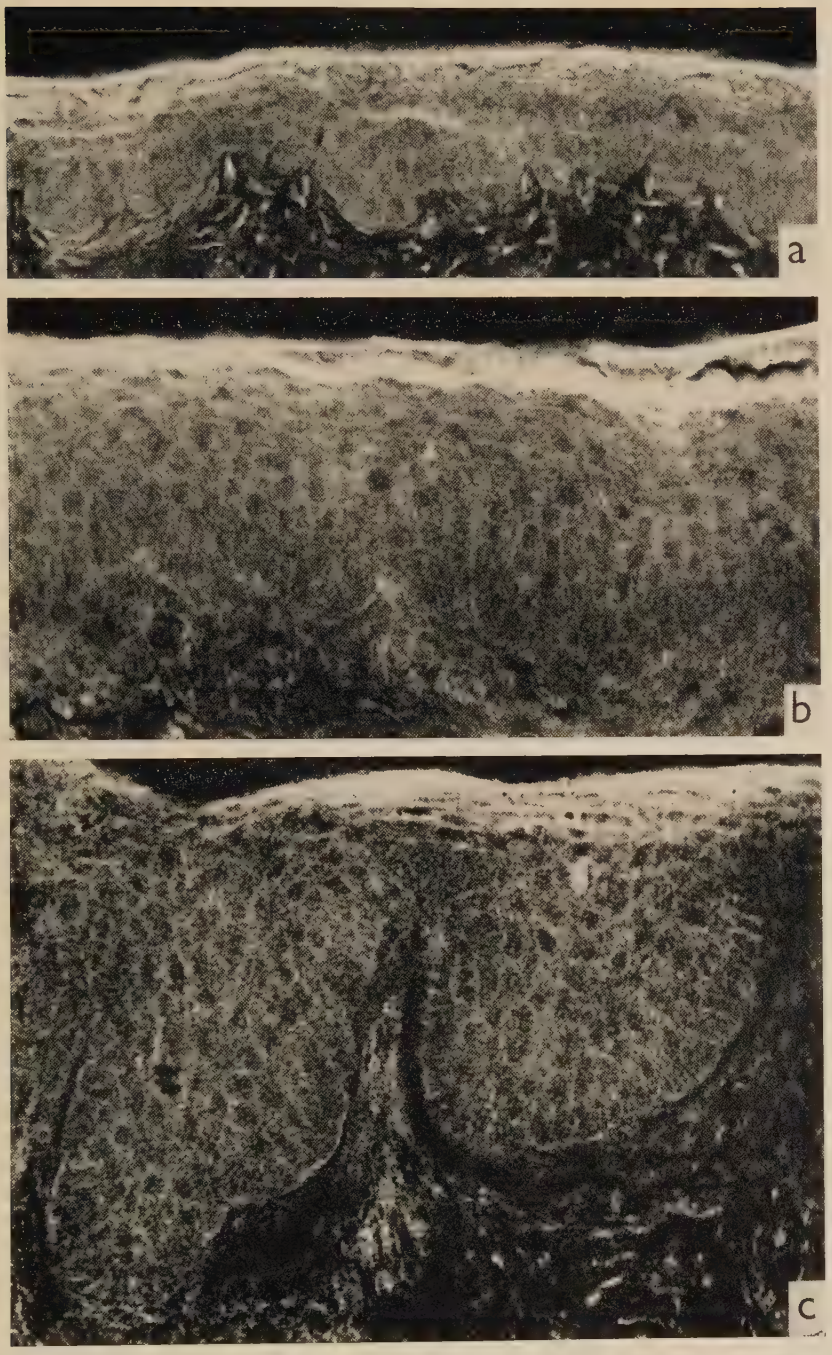
the inner layers of the cancer cords and the spinous layer of normal epithelium was 0.91 with a standard error of the mean of ± 5 per cent. Thus, when compared with the corresponding ratios obtained from frozen-dried material (0.94 and 0.66 respectively) the relative increase of the dry weight in the formalin-fixed carcinomas is apparent and more pronounced in the outer cell layers of the cancer cords. The ratio between the dry weights of the inner and the outer layers of 24 individual formalin-fixed cancer cords was 0.61 with a standard error of the mean of ± 4 per cent, while the corresponding ratio from 19 individual cords in frozen-dried specimens was 0.75 with a standard error of the mean of ± 6 per cent.

The *normal epidermis in the mouse* comprises only a few cell layers. In the non-keratinized part of this thin epithelium a uniform distribution of the dry weight was found (Fig. 3 a). In the considerably thicker *precancerous hyperplastic epithelium* (Fig. 3 b and c) as well as in the epithelium of the *non-infiltrating papillomas* (Fig. 3 d) the dry weight was also homogeneously distributed in the different layers (except for keratinized structures which had a considerably higher dry weight). A decrease in the dry weight per unit volume of about 30 per cent was found in the precancerous epidermis (formalin-fixed material) as compared with the normal epithelium. However, the dry weight values obtained from the thin normal epidermis must be considered open to relatively large errors. In the fully developed *epidermoid carcinomas* a varying distribution of the dry weight was found in the different layers of the cancer cords, similar to that found in the vaginal squamous cell carcinomas (Fig. 4).

Human material.—In the formalin-fixed *normal human epidermis* a fairly homogeneous distribution of the dry weight was found in the different cellular layers (Fig. 5). In the hyperplastic cords of the *non-neoplastic, proliferating epidermis* a higher dry weight was found in the basal cell layer than in the spinous layer (Fig. 6 a) (mean ratio between the spinous and the basal layer 0.66, the difference from 1 being highly significant with $P < 0.01$). The basal cell layer of the hyperplastic cords had about the same dry weight per unit volume as the corresponding layer in the normal epithelium. The ratio between the spinous layer of the hyperplastic and of the normal epidermis, on the other hand, was 0.63 ($P < 0.01$). Thus, a significantly decreased

---

Fig. 1. Frozen-dried vaginal mucosa of the mouse. All figures (1–9) are photo-micrographs of primary microradiograms, secondary enlargement × 350. On all figures light areas correspond to a high roentgen absorption and, hence, have a high dry weight. (*a*) Normal squamous epithelium, (*b*) Slightly hyperplastic, regenerating epithelium, (*c*) Precancerous hyperplastic epithelium.


a
b
c

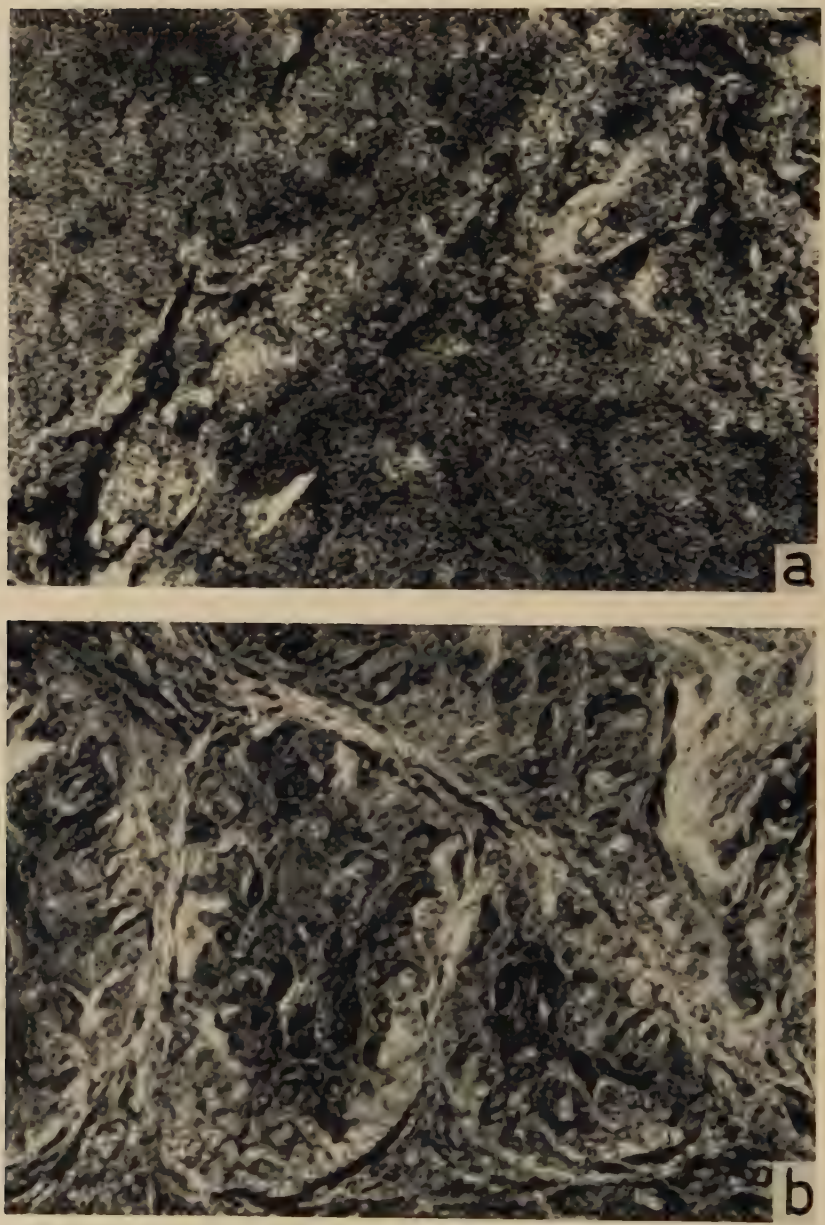


Fig. 2. Squamous cell carcinoma from the vaginal mucosa of the mouse. (*a*) Frozen-dried, (*b*) Formalin-fixed.

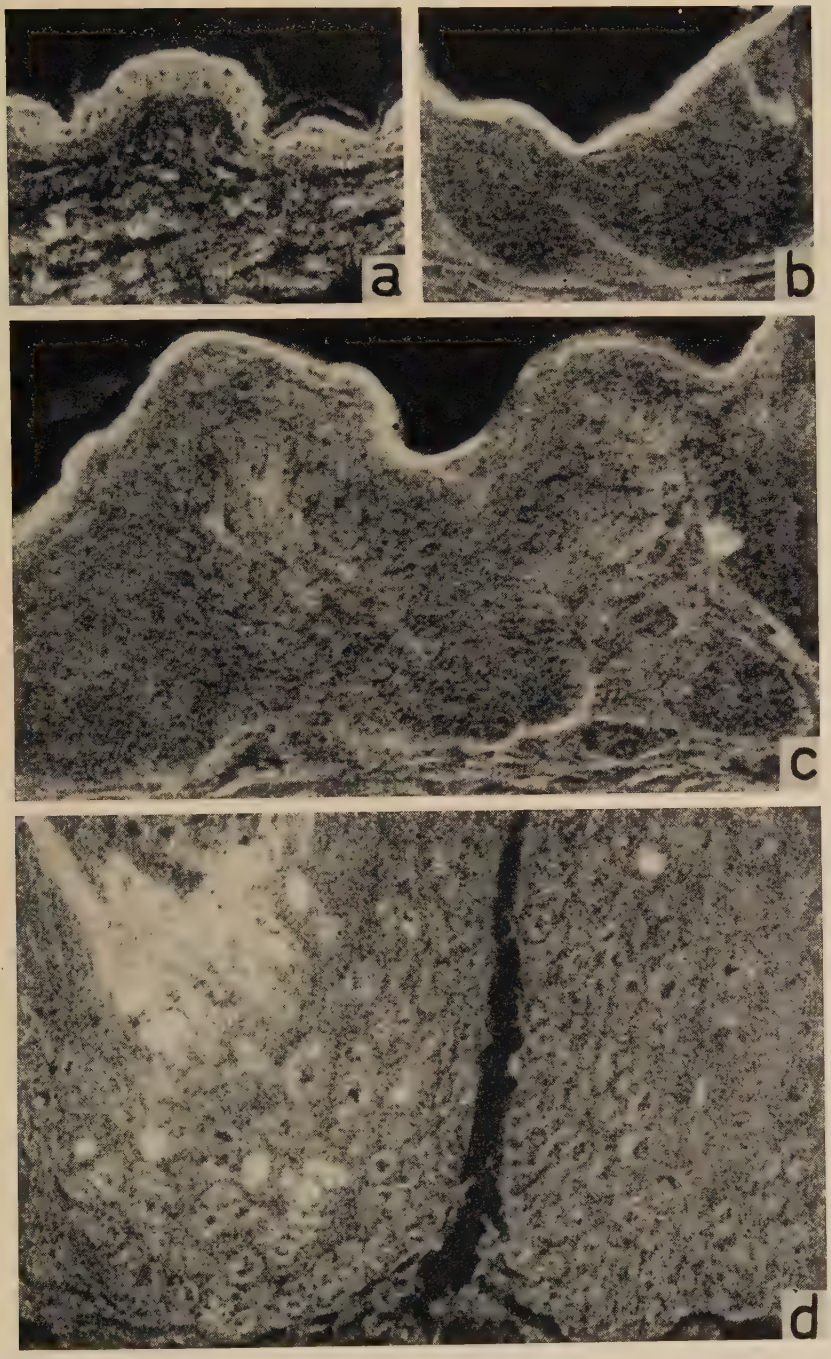


Fig. 3. Mouse epidermis. Formalin-fixed. (*a*) Normal, (*b*) and (*c*) precancerous hyperplasia, (*d*) Papillomatous, non-infiltrating tumor.

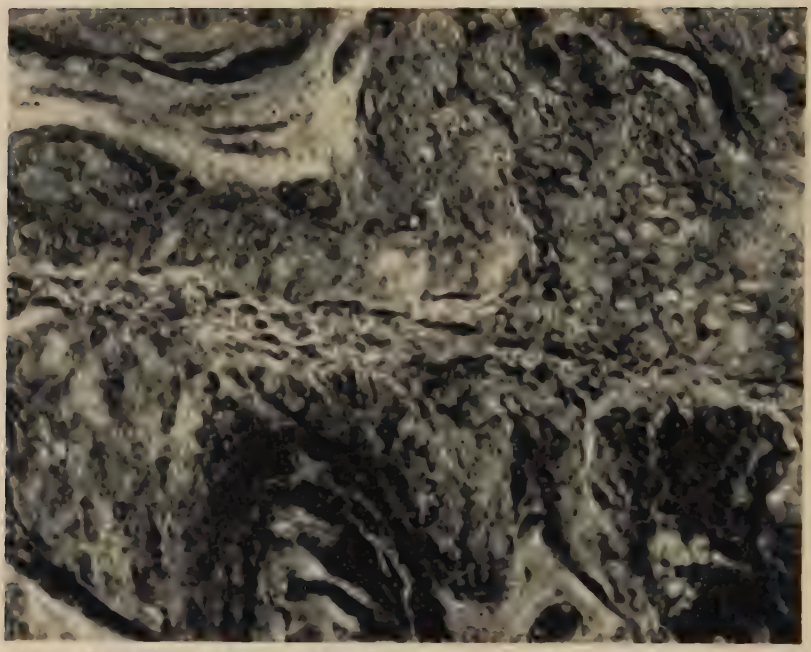


Fig. 4. Epidermoid carcinoma from the skin of the mouse. Formalin fixation.

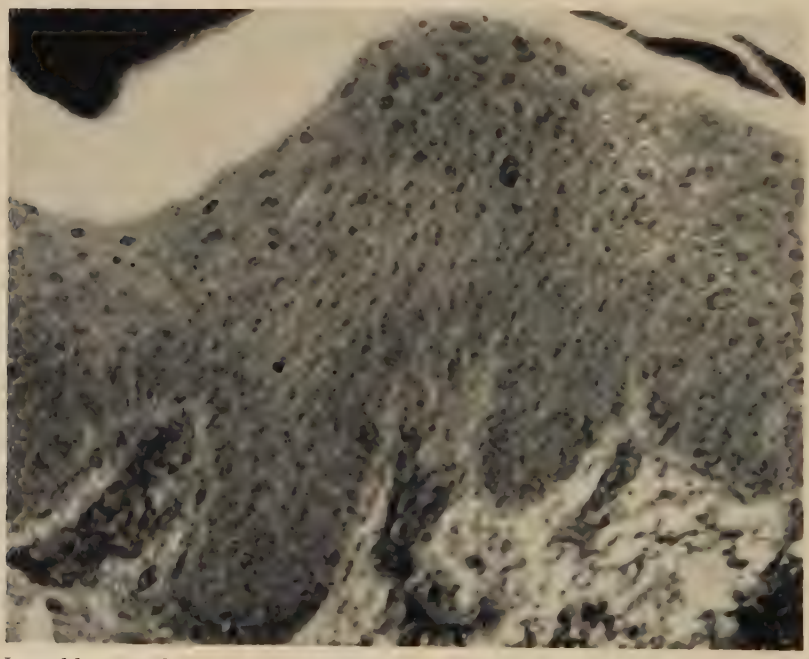


Fig. 5. Normal human plantar epidermis. Formalin fixation.

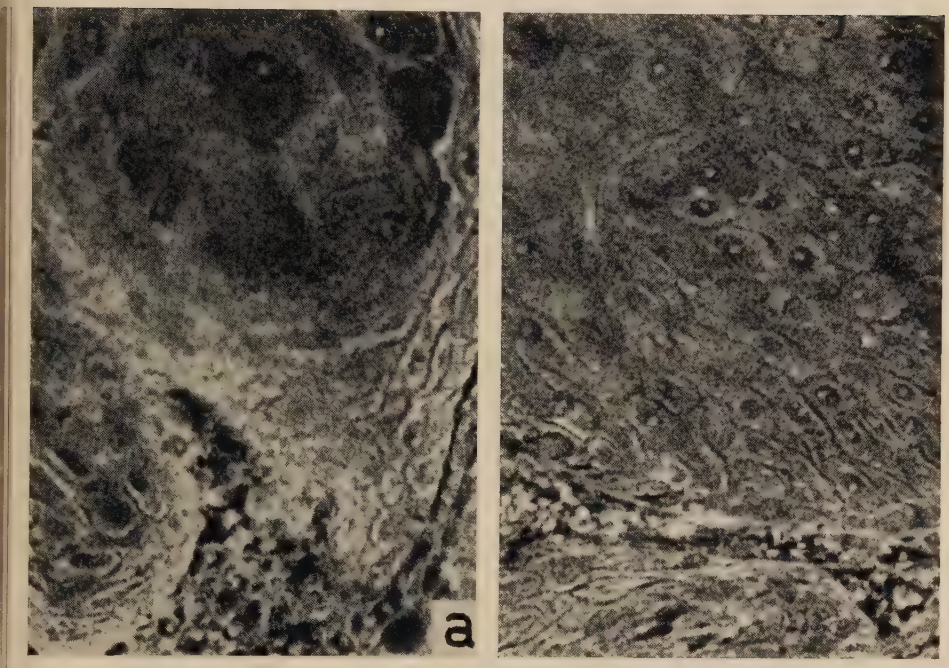


Fig. 6. Hyperplastic human epidermis. Formalin fixation. (*a*) Proliferating non-neoplastic epithelium, (*b*) Precancerous hyperplasia.

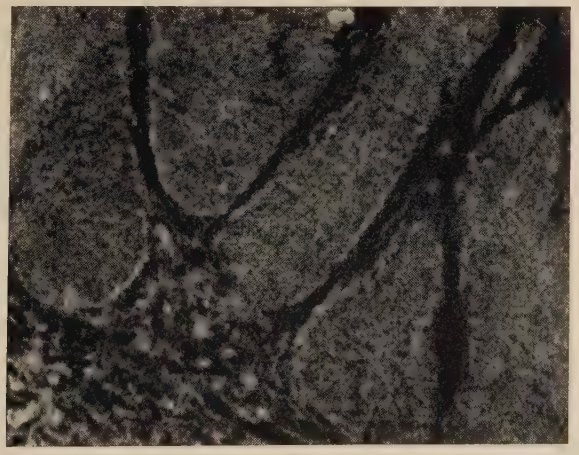


Fig. 7. Basal cell carcinoma. Formalin fixation.

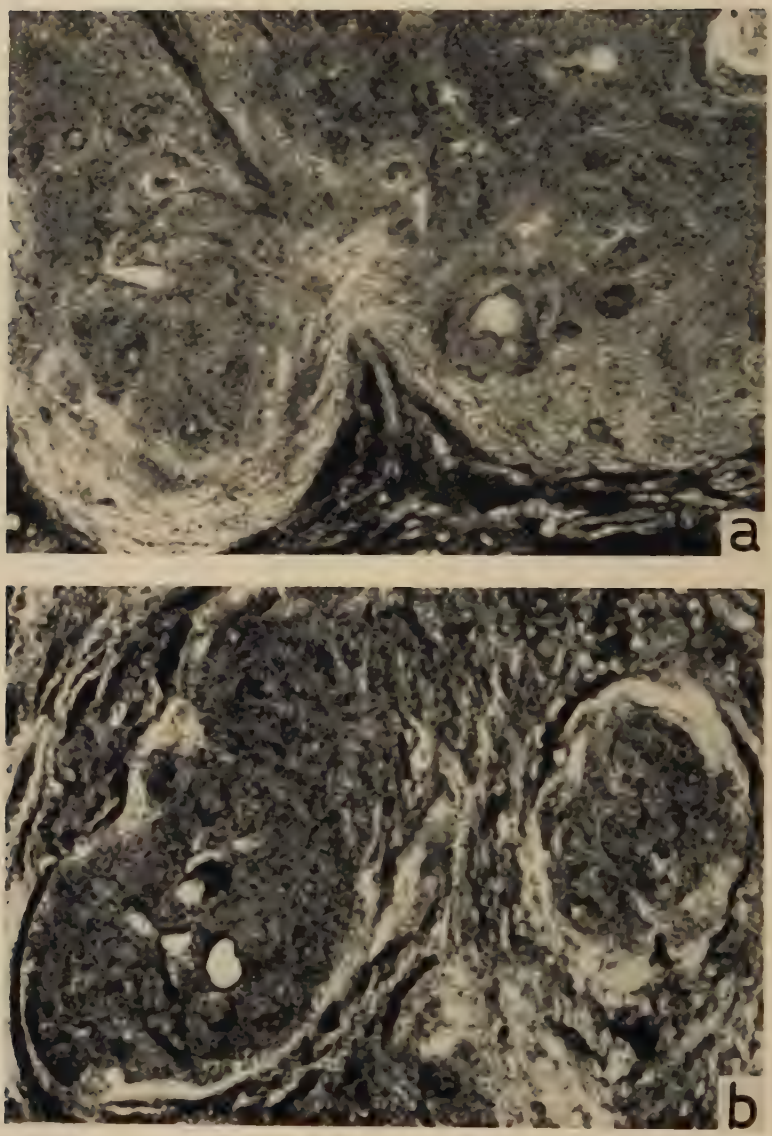


Fig. 8. Epidermoid carcinomas. Formalin fixation. (*a*) Highly differentiated, (*b*) Moderately differentiated. Note the higher dry weight in the outer than in the inner layers of the cords.

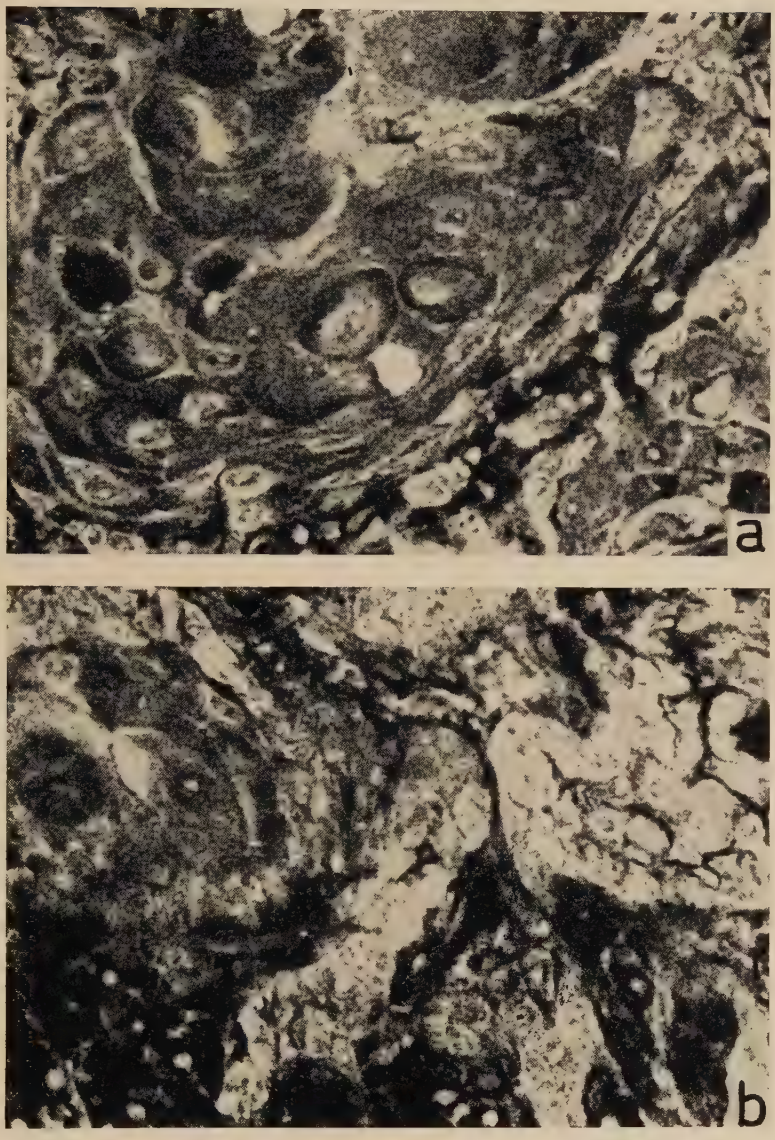


Fig. 9. Epidermoid carcinomas with a low degree of differentiation. Formalin fixation. (*a*) Cancer cord with irregular keratinization, (*b*) infiltrating cords with a relatively high dry weight as compared with the rest of the tumor.

dry weight was found in the spinous layer of the hyperplastic epidermis as compared with the corresponding layer in the normal epithelium.

Similar results were obtained from the *precancerous hyperplastic epidermis* (Fig. 6 b). In these hyperplastic cords the dry weight of the spinous layer was considerably lower than that of the basal layer (mean ratio 0.57). The dry weight of the basal layers of the hyperplastic cords was of the same magnitude as that of the corresponding layer in normal epidermis. The decrease in the dry weight of the spinous layer in the precancerous hyperplastic epidermis as compared with the same layer of the normal epithelium was of the same magnitude as in the non-neoplastic, proliferating epidermis.

In one *basal cell carcinoma*, a homogeneous distribution of the dry weight was found in the different layers of the solid cancer cords (Fig. 7). The cords had the same average dry weight per unit volume of tissue as normal epidermis.

Four *epidermoid carcinomas* with different degrees of differentiation were analysed (Fig. 8 and 9). Table I shows the mean ratios between the dry weight in the different layers of the cancer cords in these carcinomas and the dry weight in corresponding layers of normal epidermis. As can be seen from Table I, a lower dry weight was found in the inner layers of the cancer cords as compared with the spinous layers of normal epidermis. A slightly higher dry weight was observed in the outer layers of the cancer cords than in the basal layer of normal epidermis. Although in one slowly growing carcinoma a homogeneous distribution of the dry weight was found in different layers of the cords, variations in the dry weight in different parts of the tissue seemed to be charcteristic of epidermoid carcinoma as compared with normal epithelium. Furthermore, in the different layers of the cancer cords individual cells with a high dry weight were frequently found.

TABLE I

Ratios of the dry weight per unit volume in four formalin-fixed human epidermoid carcinomas (I–IV). C_o = outer layer of cancer cords, C_i = inner layer of cancer cords, N_b and N_{sp} = basal and spinous layer of normal epidermis respectively.

Case No.	Mean ratios		
	C_o/N_b	C_i/N_{sp}	C_i/C_o
I	1.09	0.70	0.64
II	1.22	0.59	0.51
III	1.07	0.55	0.51
IV	1.08	0.91	0.83

DISCUSSION

Roentgen absorption spectrophotometry [12, 14] was applied to *quantitative* determination of the dry weight of the different layers of squamous epithelium during carcinogenesis. The experimental conditions were arranged so that ratios between dry weights per unit volume of tissue in different structures were obtained without measuring the thickness of the tissue sections.

A homogeneous distribution of the dry weight was found in the different layers of the normal vaginal epithelium of the mouse. The dry weight was also uniform in the epidermis which, however, was too thin to permit the identification of individual layers. A significantly lower dry weight was found in the frozen-dried, non-neoplastic, regenerating, slightly hyperplastic vaginal epithelium as compared with the normal epithelium (decrease about 20 per cent). In the frozen-dried hyperplastic vaginal epithelium during early carcinogenesis the dry weight was between 25 and 30 per cent lower than in normal epithelium. A similar decrease in dry weight was also found in precancerous, hyperplastic epidermis as compared with the normal epithelium. A greater variability of the dry weight was observed in different parts of fully developed squamous cell carcinomas of vaginal origin as well as in epidermoid carcinomas. The outer cell layers of the cancer cords in frozen-dried carcinomas had about the same dry weight as the basal layer of normal epithelium. The inner layers of the cancer cords, on the other hand, had a dry weight which was 30 to 35 per cent lower than that of the spinous layers of the normal epithelium. Thus a considerable difference between the dry weight of the outer and the inner layers of the cancer cords could be established in frozen-dried material. This was mainly caused by a decreased dry weight of the inner layers.

The use of fixatives, such as neutral formalin, for the preservation of the tissues for the absorption analyses was considered to affect the distribution of the dry weight in the tissue structures. Protein material may be dissolved out by the fixative [24, 26] and contraction of the specimens during the fixation procedure [24] may increase the dry weight per unit volume of tissue.

In formalin-fixed material a considerably higher dry weight was generally found than in frozen-dried specimens (increase varying between 30 and 100 per cent). In formalin-fixed squamous cell carcinomas, a somewhat higher ratio was found between the dry weight of the outer and the inner layers of the cords than in frozen-dried tissues. A rather varying degree of contraction of different structures probably accounts for this higher ratio in the

formalin-fixed carcinomatous tissues. Furthermore, losses of substances such as protein material during the fixation procedure seem to be of less importance than contraction as far as the determination of dry weight of the tissue is concerned. The generally increased and variable dry weight of different structures caused by a varying degree of contraction during the formalin fixation makes such material unsuitable for quantitative histochemical studies unless these are performed on a dry weight basis. Relative measurements of adjacent structures can be considered to be more reliable.

The same general results were obtained from the human, formalin-fixed tissues as from the experimental, frozen-dried and formalin-fixed material. In one basal cell carcinoma the same dry weight was found in the different layers of the cancer cords as in normal epidermis. In the epidermoid carcinomas considerable variations in the dry weight were found in the different layers of the cancer cords. Often but not always there was an increased dry weight of the outer cell layers and a decreased dry weight of the inner layers of the cancer cords as compared with normal epidermis. A greater difference between the dry weight in different layers of the tissue appeared to be characteristic of epidermoid carcinomas. This was in contrast to the fairly homogeneous distribution of the dry weight found in the different layers of normal epidermis.

The quantitative changes in the distribution of the dry weight of squamous epithelium during carcinogenesis established by means of roentgen absorption spectrophotometry confirm the general results obtained from microchemical analyses (for reviews see [3] and [8]). The decreased dry weight of the regenerating and precancerous, hyperplastic epithelium as compared with normal epithelium agrees well with the decreased protein-nitrogen [23] the general demineralization [22], and the increased water content [25] revealed by microchemical analyses of mouse epidermis during early carcinogenesis. Thus, the considerably lower calcium content (decrease about 40 per cent) in the non-neoplastic and precancerous hyperplastic epidermis [3] is of about the same magnitude as the decrease of the dry weight found in the present investigation.

The experimentally induced epidermoid carcinomas have a still higher water content than normal and hyperplastic epidermis as revealed by microchemical analyses [25]. This corresponds well with the marked decrease in the dry weight of the inner layers of the cancer cords. These inner layers constitute a greater portion of the total volume of the carcinomatous tissue than the outermost cell layers [13]. Therefore, changes in the composition of the inner layers have a predominant influence on chemical determinations

involving the whole mass of tumor. A varying amount of keratinized material, with its high dry weight may also have a considerable influence on the results of chemical analyses on pooled samples of epidermal tissue.

Continuous growth of the squamous epithelium takes place throughout the life of the adult organism. During this process, a progressive increase in cell volume occurs towards the superficial layers. The homogeneous distribution of the dry weight found in the various kinds of normal squamous epithelium indicates that changes in the total weight of the individual cell approximately follow the changes in its volume [19]. During carcinogenesis there is an increase in the volume of the basal and the spinous cells [9]. This has been attributed to a higher water content of the individual cells [25]. The decreased dry weight of the hyperplastic epithelium as compared with the normal, demonstrated in this paper, may suggest that during early carcinogenesis, the increase in cell volume may be due only to an increase in the water content of the hyperplastic cells. In order to elucidate whether this was the case, a comparative study of the changes in the dry weight and in the cell volumes of the same structures was performed. Absolute cell volumes, however, cannot be accurately measured in epidermal tissues because of the indistinct cell boundaries. Therefore, the relative cell volumes in normal and hyperplastic epithelium were determined. The relative volume of individual cells in different structures was obtained from the reciprocal of the number of nuclear centers per unit volume of the tissue section. According to Abercrombie [1],

$$n_1 = k_1(d_1 + 2r_1)y,$$

where n_1 = number of nuclear fragments, k_1 = number of cells per unit volume of tissue, d_1 = thickness of the section, r_1 = mean nuclear radius, and y = a given area. Because of the varying size of the nuclei in different structures, comparisons of the correct number of cells in these can be made from determinations of differences between the number of nuclear fragments in sections with different thicknesses. Thus, for the same structure in a section with a different thickness, $d_2 < d_1$, another number of nuclear fragments, n_2, will be obtained according to:

$$n_2 = k_1(d_2 + 2r_1)y.$$

Subtraction of the latter equation from the former gives:

$$n_1 - n_2 = k_1(d_1 - d_2)y.$$

For a different structure in the same pair of sections the corresponding equation holds:

$$n_3 - n_4 = k_2(d_1 - d_2)y.$$

The ratio of the number of nuclear centers per unit volume in the two structures is then obtained as:

$$\frac{n_1 - n_2}{n_3 - n_4} = \frac{k_1}{k_2}.$$

$\frac{k_2}{k_1}$ is then the ratio between the cell volumes in the two structures.

In normal and hyperplastic human epidermis, embedded together and cut from the same block of paraffin, the relative volumes of the basal and the spinous cells were determined from countings of nuclear centers in consecutive sections with different thicknesses ($\sim 10\mu$ and $\sim 5\mu$ respectively). The ratio between the volumes of the spinous and the basal cells in normal epidermis was between 1.7 and 2, whereas in the hyperplastic epidermis the corresponding ratio was between 5 and 6. Since in the normal epidermis the dry weight per unit volume was about the same in the basal and in the spinous layers, the total weight of the individual spinous cells could be considered to be 1.7 to 2 times that of the basal cells. As the dry weight per unit volume in the spinous layer of this hyperplastic epithelium was about 40 per cent of that in the basal layer, the hyperplastic spinous cells could be calculated to have a total weight 2 to 2.5 times as high as that of the basal cells. Furthermore, as the dry weight of the spinous layer in the hyperplastic epidermis was about 50 per cent of that of the corresponding layer in the normal epidermis and as the ratio between the cell volumes in the two epithelia was between 3 and 4, the individual spinous hyperplastic cells had an average total weight between 1.5 and 2 times that of the normal spinous cells. In spite of the decreased dry weight of the spinous layers in the hyperplastic epithelium, the total weight of the individual hyperplastic spinous cells thus was considerably higher than that of the corresponding normal cell. Thus, an increase in cell volume during the hyperplastic stage of carcinogenesis can only partly be ascribed to an increased water content. It must also be due to a considerable accumulation of organic material, mainly proteins, in these cells.

Variations in the dry weight per unit volume in different structures, i.e. the concentration of organic material, mainly proteins, may considerably influence the histochemical appearance of the structures. Thus, in epider-

moid cancer cords the outer cell layers with a high dry weight often had a high affinity for basic dyes [19]. However, acid compounds, such as nucleic acids, are mainly responsible for the basophilic properties of different structures [2]. A high ultraviolet absorption at about 260 mμ of the cells in the outer layers of infiltrating cords in a number of epithelial tumors [7] as well as in epidermoid carcinomas [17] indicates the presence of nucleic acids in high concentrations in these cells. High concentrations of proteins may, however, also contribute to the total extinction values in spite of the high selective absorption of nucleic acids in this wave length range. Therefore, the significantly higher dry weight of the outer than of the inner layers of the cords in formalin-fixed squamous cell carcinomas, revealed by the roentgen absorption analyses in the present investigation, could contribute to the high ultraviolet absorption obtained from corresponding cells. On the present material a comparative study of the dry weight and the ultraviolet absorption in the *same* structures, measured by scanning microspectrophotometric procedures [4, 5, 6] was also performed [18]. The investigation showed that the mean extinctions at about 260 mμ, measured in cytoplasmic areas, per unit of dry weight generally was higher in the outer than in the inner layers of the cancer cords and also higher than in the basal layer of the homologous normal epithelium. The results thus support the concept, that a high ultraviolet absorption at about 260 mμ in the outer layers of cancer cords is mainly attributable to the presence of substances with high extinction coefficients at this wave length, i.e. nucleic acids. From comparative roentgen and ultraviolet absorption measurements on nerve cells [11, 21] a correlation has been suggested between the dry weight and the absorption capacity in ultraviolet. The same conclusions have been drawn from interference microscopical studies on a few formalin fixed human carcinomas of different origin [10] which conclusions, however, were not supported by any ultraviolet measurements. In the present material, keratinized structures with a high dry weight had a rather low absorption in ultraviolet at about 260 and 280 mμ, whereas, in contrast, the underlying epithelium had a much lower dry weight but a rather high ultraviolet absorption in the same wave lengths [18]. In epidermal tissues and derived tumors, thus, no correlation between the absorption of soft continuous roentgen rays (dry weight) and the ultraviolet absorption was demonstrable in structures with a different chemical composition.

In the present investigation a significant decrease of the dry weight was established in the spinous layer of the hyperplastic squamous epithelium during carcinogenesis as compared with normal epithelium. Nevertheless,

the individual spinous, hyperplastic cells had a total weight considerably exceeding that of the corresponding normal cells. In the fully developed squamous cell carcinomas the outer cell layers in the infiltrating cords generally had a higher dry weight than the inner layers. However, in frozen-dried material, the quantitative roentgen absorption measurements revealed no significant difference between the dry weight of the outer layers in the cancer cords and the basal layer in homologous normal epithelium. Formalin fixation has a pronounced effect on the distribution of dry weight of different structures and is therefore unsuitable for quantitative histo- and cytochemical investigations unless these are performed on a dry weight basis.

SUMMARY

Quantitative roentgen absorption spectrophotometry was applied to the determination of the dry weight of the different layers of squamous epithelium during carcinogenesis. The investigation revealed definite changes in the distribution of the dry weight of different structures during the development of experimentally induced malignant tumors. Corresponding variations in the dry weight were also found in some human biopsy specimens, representing various stages from normal epidermis to fully developed epidermoid carcinomas. A comparison of the dry weight in frozen-dried and formalin-fixed specimens was made and showed the pronounced effect of formalin fixation upon the dry weight of different structures. The results are discussed in relation to earlier micro- and histochemical investigations.

REFERENCES

1. Abercrombie, M., *Anat. Rec.* **94**, 239 (1946).
2. Atkinson, W. B., *Science* **116**, 303 (1952).
3. Carruthers, C., *Cancer Research* **10**, 255 (1950).
4. Caspersson, T., *Exptl. Cell Research* **1**, 595 (1950).
5. Caspersson, T., Jacobsson, F., and Lomakka, G., *ibid.* **2**, 301 (1951).
6. Caspersson, T., Lomakka, G., and Svensson, G., *ibid.* **7**, 601 (1954).
7. Caspersson, T. and Santesson, L., *Acta Radiol.* Suppl. **46** (1942).
8. Cowdry, E. V., *Advances in Cancer Research* **1**, 57 (1953).
9. Cowdry, E. V. and Paletta, F. X., *J. Natl. Cancer Inst.* **1**, 745 (1941).
10. Davies, H. G. and Engfeldt, B., *Lab. Invest.* **3**, 277 (1954).
11. Engström, A., *Discussions Faraday Soc.* **9**, 427 (1950).
12. Engström, A. and Lindström, B., *Biochim. et Biophys. Acta* **4**, 351 (1950).
13. Glucksman, A., *Cancer Research* **5**, 385 (1945).
14. Lindström, B., *Exptl. Cell Research* **6**, 537 (1954).
15. —— *Acta Radiol., Suppl. 125* (1955).
16. Lindström, B. and Moberger, G., *Exptl. Cell Research* **6**, 540 (1954).
17. Moberger, G., *Acta Unio Intern. contra Cancrum* **9**, 4 (1953).
18. —— *Acta Radiol.*, Suppl. **112** (1954).

19. Moberger, G. and Engström, A., *J. Invest. Dermatol.* **22**, 477 (1954).
20. Moberger, G., Lindström, B., and Andersson, L., *Exptl. Cell Research* **6**, 228 (1954).
21. Nurnberger, J., Engström, A., and Lindström, B., *J. Cellular Comp. Physiol.* **39**, 215 (1952).
22. Paletta, F. X., Cowdry, E. V., and Lischer, C. E., *Cancer Research* **1**, 942 (1941).
23. Roberts, E. and Frankel, S., *ibid.* **9**, 231 (1949).
24. Stowell, R. E., *Stain Technol.* **16**, 67 (1941).
25. Suntzeff, V. and Carruthers, C., *Cancer Research* **6**, 574 (1946).
26. Sylvén, B., *Acta Unio Intern. contra Cancrum* **7**, 708 (1951).
27. Winzler, R. J., Physiopathol. of Cancer. Hoeber and Harper, New York, 1953.

L'ULTRASTRUCTURE DU NUCLÉOLE DE CELLULES NORMALES ET CANCÉREUSES

ÉTUDE AU MICROSCOPE ÉLECTRONIQUE

W. BERNHARD, ANTOINETTE BAUER, A. GROPP[1], FRANÇOISE HAGUENAU et CH. OBERLING

Institut de Recherches sur le Cancer, Villejuif (Seine), France

Reçu le 25 septembre 1954

Depuis longtemps déjà, certains auteurs avaient imaginé que le nucléole pouvait avoir une structure particulière et l'on retrouve de nombreuses descriptions de nucléoles hétérogènes dès la fin du siècle dernier (Flemming, Lenhossek, Levi, Ruzicka, Simmaro, etc. . . .). On décrivait des structures alvéolaires (Purcell, Schaudinn) ou réticulées (Butschli, Leydig).[2]

En fait, il s'agissait essentiellement de vacuoles ou de sphérules intranucléolaires. La notion de l'existence d'un filament nucléolaire est en réalité très récente.

Si nous laissons de côté tout ce qui concerne la cellule végétale, l'histoire de l'appareil filamenteux remonte aux travaux de Ramón y Cajal qui, grâce à ses méthodes d'imprégnation à l'argent, décrit un nucléole complexe, filamenteux, dans les glandes digestives de larves de Diptères. Mais après avoir décrit cette structure (1893), il hésite à exclure formellement la possibilité d'artéfacts (1921). En 1930, un auteur uruguayen, C. Estable [8], décrit, au niveau des cellules nerveuses, un nucléole à structure compliquée, filamenteux et glomérulaire. Avec J. R. Sotelo, Estable publie en 1951 [9] un très important travail d'ensemble sur la question du nucléole filamenteux. Les auteurs uruguayens, sur un matériel très varié (ovocytes d'Invertébrés, cellules nerveuses de Vertébrés, cellules végétales, etc. . . .) utilisent plusieurs techniques et obtiennent des images filamenteuses impressionnantes, non seulement après argentation (technique d'Estable), mais aussi *in vivo*, au contraste de phase. Ils concluent que dans toutes les cellules animales et végétales le nucléole est composé de deux substances distinctes: l'une toujours présente, filamenteuse, qu'ils dénomment le « *nucleolonema* », l'autre, homogène et variable, la « *pars amorpha* ».

[1] Travail effectué au cours d'un stage rendu possible par une bourse de l'O.M.S.
[2] Littérature dans: Vigier [14], Estable et Sotelo [9] et C. et O. Vogt [15].

La même année, le microscope électronique permet à son tour de découvrir cet aspect si particulier. Presque simultanément et indépendamment, Borysko et Bang en Amérique [3], Bernhard, Haguenau et Oberling en France [1], publient des images de nucléoles où leur structure complexe et filamenteuse apparaît de façon spectaculaire.

La diversité du matériel utilisé permettait de penser que ces constatations pouvaient s'étendre à la plupart des cellules. C'est pourquoi nous avons poursuivi nos recherches tant sur le matériel déjà utilisé lors de notre premier travail que sur d'autres tissus normaux. De plus, comme l'on sait que l'hypertrophie nucléolaire est un des signes les plus caractéristiques de la cellule maligne, nous avons étudié aussi les tissus cancéreux afin de savoir s'il existait des différences entre les nucléoles de la cellule normale et ceux de la cellule cancéreuse.

Notre examen a ainsi porté sur des *tissus normaux* de rat (foie, rein, hypophyse, placenta de rate entre le 10ème et le 14ème jour suivant une suggestion du Professeur Mayer de Bordeaux), et sur des *tissus tumoraux*:

— chez le *rat*: hépatome induit par le jaune de beurre, épithélioma utérin (T. 8).

— chez la *souris*: tumeur mammaire (T. 120) et tumeur ascitique d'Ehrlich.

— chez le *lapin*: fibrome et fibrosarcome de Shope.

Enfin, un cancer humain (épithélioma glandulaire du sein) a pu être étudié.

Pour la plupart de ces recherches, nous avons effectué des coupes ultrafines selon la technique utilisée à l'heure actuelle dans notre Laboratoire : fixation des tissus pendant une heure dans l'acide osmique à 1 % tamponné suivant la méthode de Palade, déshydratation dans les alcools et inclusion dans le methacrylate de butyle. Les coupes ont été faites à l'aide du microtome Servall-Porter. Leur épaisseur varie entre 200 et 400 Å. Quelques prélèvements ont été inclus dans l'Esterwax et coupés au microtome Rocking, Cambridge. Ces coupes sont sensiblement plus épaisses (1.000 à 2.000 Å, Fig. 1 et 2). Les photos ont été prises, soit au microscope électronique RCA, type E.M.U. 2E, soit au microscope électronique Trüb-Täuber.

Les résultats obtenus ont confirmé, dans l'ensemble, notre première étude: quelle que soit l'origine des cellules, leurs nucléoles ont le plus souvent un *aspect pelotonné* dans les coupes relativement épaisses (0,1–0,2 μ) (Fig. 1 et 2), et *réticulaire* dans les coupes très fines (Fig. 3 à 10).

Ces structures sont particulièrement bien visibles quand les *nucléoles sont hypertrophiés*, soit dans des cellules normales: dans le foie en régénération et dans des cellules choriales de placenta par exemple, soit dans des

cellules cancéreuses. Le diamètre du filament qui constitue ce réseau, mesure au minimum 450 Å, au maximum 2.000 Å. Nous sommes enclins à penser que les tronçons séparés les uns des autres, à l'intérieur du nucléole, représentent en général les portions d'un réseau ou d'un filament continu, artificiellement séparées par la coupe.

Dans notre première note, nous nous sommes demandés si les structures réticulaires étaient dues à une *coalescence* réelle des filaments ou à une *superposition optique* de structures indépendantes les unes des autres. Or, les coupes les plus minces, d'environ 200 Å d'épaisseur, excluent cette deuxième hypothèse. On y peut facilement observer, aux points de contact des mailles du réseau, l'existence d'une continuité dans la structure fine des différents filaments. Notons d'autre part, qu'il existe parfois, en quelques points du nucléole des renflements irréguliers de ces filaments. Enfin, au cours de ces observations, nous n'avons jamais trouvé jusqu'ici une striation transversale comparable à celle des chromosomes de Diptères.

En plus de ces filaments nucléolaires, Estable et Sotelo [9] ainsi que Borysko et Bang [3] avaient signalé l'existence d'une *matrice homogène* dans certains nucléoles. Nous pouvons confirmer cette observation.

Dans le foie et le placenta, par exemple, les nucléoles paraissent purement filamenteux, au contraire, dans le pancréas et dans certaines tumeurs (Tumeur d'Ehrlich) ils semblent le plus souvent retenir entre les mailles de leur réseau une substance homogène qui, parfois, diffuse dans le nucléoplasme.

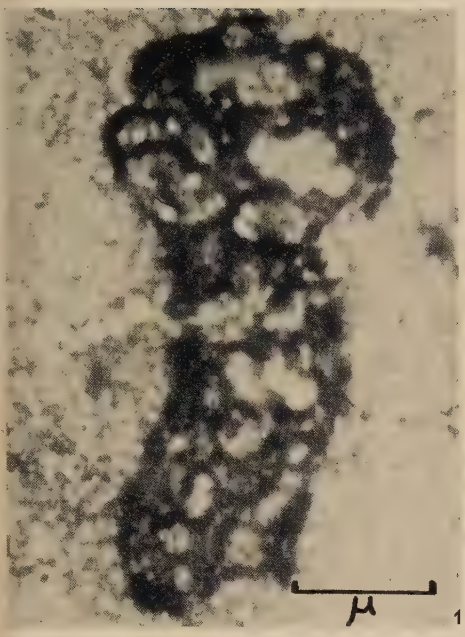
Exceptionnellement, dans les tissus de mammifères, les nucléoles peuvent être complètement homogènes, nous en avons observés dans des cellules de la Tumeur d'Ehrlich. Enfin, ils sont tous homogènes dans la glande séricigène des vers à soie (Legay et Bernhard, travail non publié). Nous ne savons pas encore si un nucléole purement filamenteux peut élaborer

---

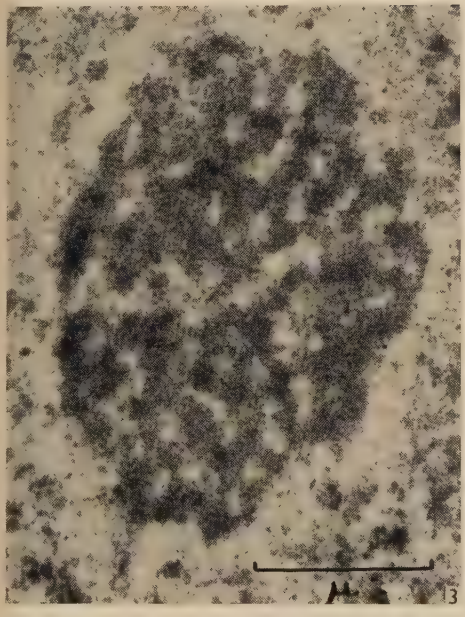
Fig. 1 et 2. *Nucléoles de cellules choriales* (placenta de rat au 14ème jour). Leur constituant filamenteux est bien visible. La figure 1 présente un aspect réticulé. Sur la fig. 2, il s'agit d'un véritable peloton dense. Inclusion à l'Esterwax. Epaisseur des coupes : 1.000–2.000 Å. Gross : 20 000 ×.

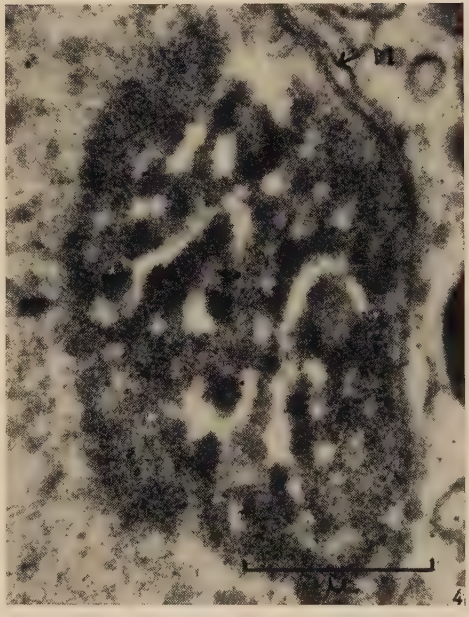
Fig. 3. *Nucléole d'une cellule hépatique normale de rat.* Aspect filamenteux facilement reconnaissable, mais on distingue sur cette coupe plus fine que la précédente (200–300 Å) les éléments granulaires qui le constituent. Gross : 24 000 ×.

Fig. 4. Autre image de *nucléole d'une cellule normale* (pancréas de rat). Le nucléole est accolé à la membrane nucléaire (→ *M*). Son aspect est le même que celui de l'image précédente, mais certaines zones du filament sont plus denses que d'autres. Il est difficile de voir exactement ici si l'on est en présence seulement d'un « nucleolonema » ou si certaines plages plus diffuses ne sont pas de la « pars amorpha ». Gross 26 000 ×.


μ
1

μ
2


μ
3


μ
4

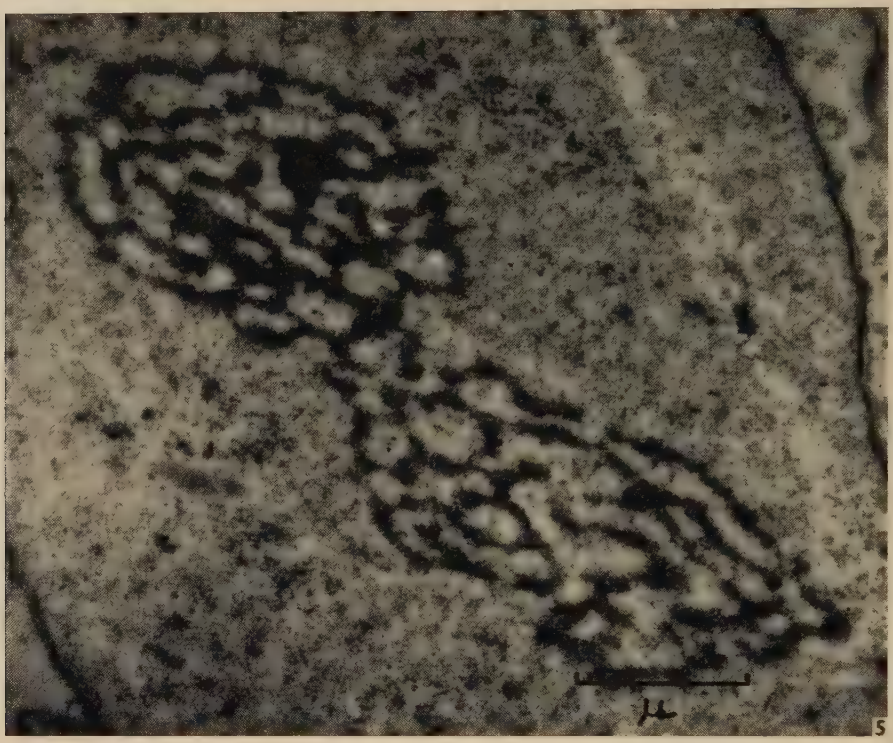


Fig. 5. *Nucléole d'une cellule du Fibrome de Shope* (lapin). La minceur du « nucleolonema » permet de voir nettement le caractère filamenteux de ce nucléole. On voit également les fins granules de sa structure ultrafine. On remarque épars dans le nucléoplasme des granules du même type. Gross : 22 000 ×.

entre ses mailles la substance homogène, s'il peut s'homogénéiser entièrement pendant certaines phases de son activité, ou s'il est, dans certains cas, homogène d'emblée.

Nous ne connaissons pas non plus le comportement du filament, avant, pendant et après la mitose.

Grâce à l'amélioration considérable des techniques de coupe depuis notre première note et grâce à l'utilisation d'un microscope électronique de haute résolution, il a été possible d'analyser en détail à la fois l'*ultrastructure* du constituant filamenteux et de la substance homogène. Dans tous les cas, le *filament* ou réseau nucléolaire est composé de grains. Ces grains, arrondis ou ovalaires, d'aspect très dense, mesurent entre 100 et 150 Å de diamètre. Ils s'accolent les uns aux autres formant parfois de courts bâtonnets qui ne paraissent pas présenter d'orientation particulière.

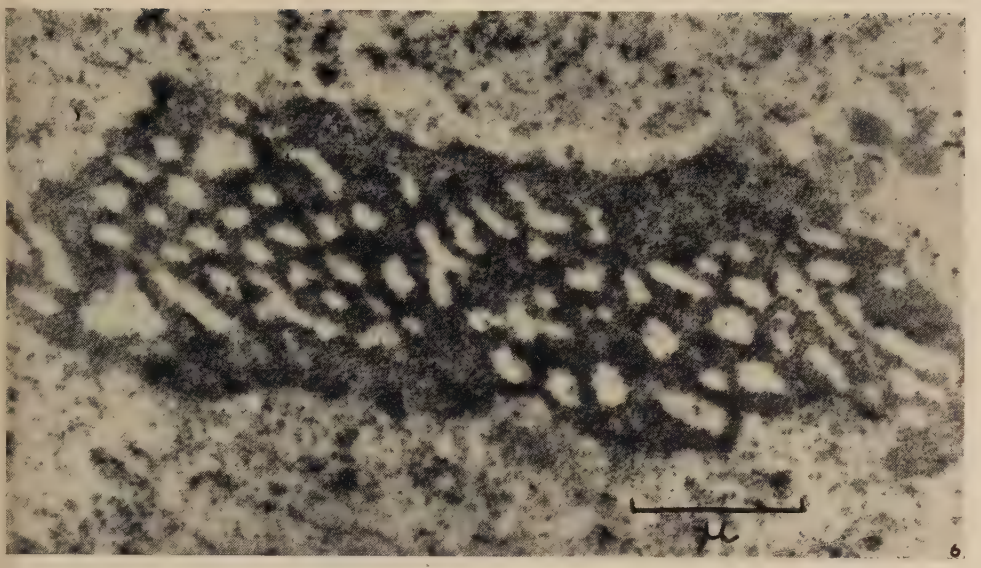


Fig. 6. Même préparation. Aspect réticulé avec des régions plus diffuses. Composants granulaires bien visibles. Gross : 22 000 ×.

On les trouve aussi enrobés séparement dans une masse moins contrastée qui semble être composée par d'autres particules allongées assez mal définies puisqu'elles se trouvent à la limite du pouvoir séparateur de notre instrument (Fig. 9 et 10). Parfois certains de ces grains se voient en dehors du filament, entre les mailles du réseau ou même dispersés dans le nucléoplasme. Souvent, il est possible d'observer des nuages de granulations identiques accolés à la surface interne de la membrane nucléaire (Fig. 7).

La *substance homogène* remplit les mailles du réseau nucléolaire ou forme un halo autour de celui-ci. Elle est constituée par les mêmes éléments mais répartis de façon plus diffuse et moins dense (Fig. 7).

Notons que ces grains arrondis et que ces particules ont approximativement la taille des grandes molécules. Il convient donc d'être très prudent dans l'interprétation de ces faits, puisque même la meilleur fixation peut révéler des artéfacts, pour le moment peu contrôlables à cette échelle. Mais, cette réserve faite, signalons que la taille et l'aspect morphologique des grains arrondis dans le filament nucléolaire et le nucléoplasme paraissent très semblables à ceux décrits par Palade [12], par Sjöstrand [13] et par nous-mêmes [2] dans le cytoplasme de cellules très basophiles : grains qui d'après ces auteurs représenteraient des molécules de ribonucléoprotéines.

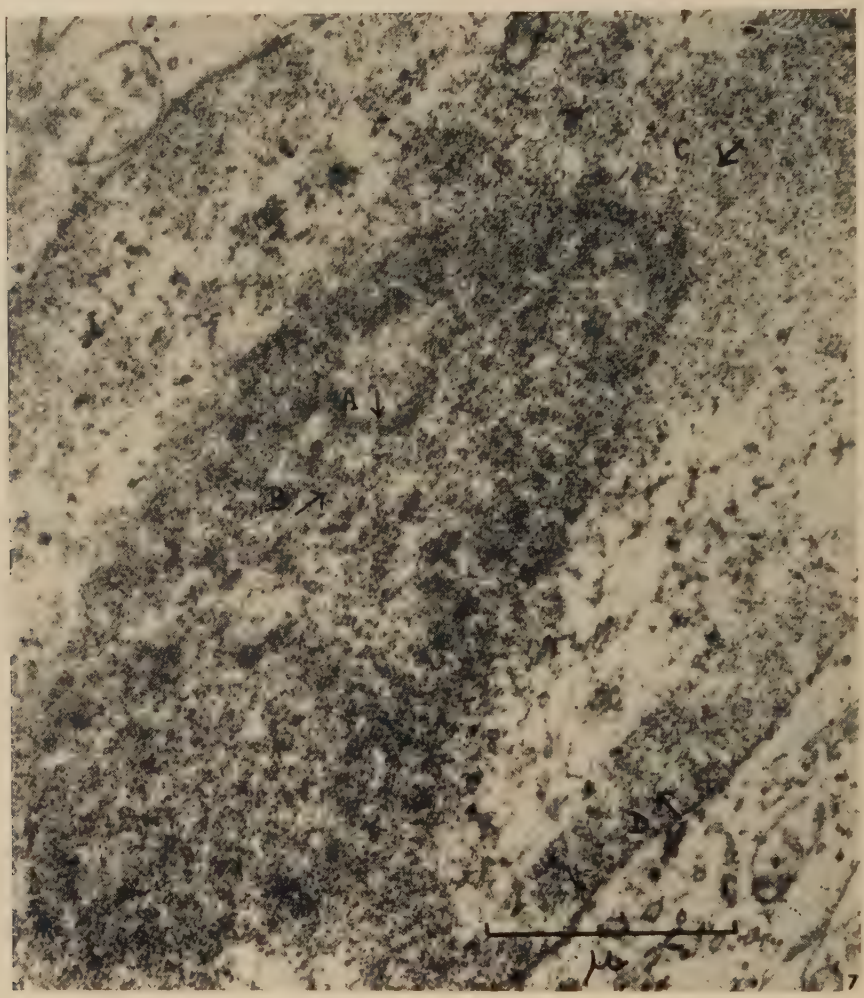


Fig. 7. Autre aspect du nucléole que l'on rencontre fréquemment dans la Tumeur ascitique d'Ehrlich. Le « nucleolonema » est peu développé. Seuls quelques tronçons ont été intéressés par la coupe (➢ *A*). Entre eux, on trouve les granules du « nucleolonema » plus dispersés (→ *B*) et mêlés à de très petites particules allongées. Ces éléments se répandent en nuage dans la région périnucléolaire (« pars amorpha » : → *C*). On les retrouve aussi accolés à la membrane nucléaire (→*D*). Gross : 32 000 ×.

Toutes les études entreprises jusqu'ici tendent à démontrer que cette ultrastructure qui vient d'être décrite est pratiquement la même pour les nucléoles de cellules normales et de cellules cancéreuses. Aucune différence constante ne paraît distinguer les uns des autres. Si la taille du nucléole de

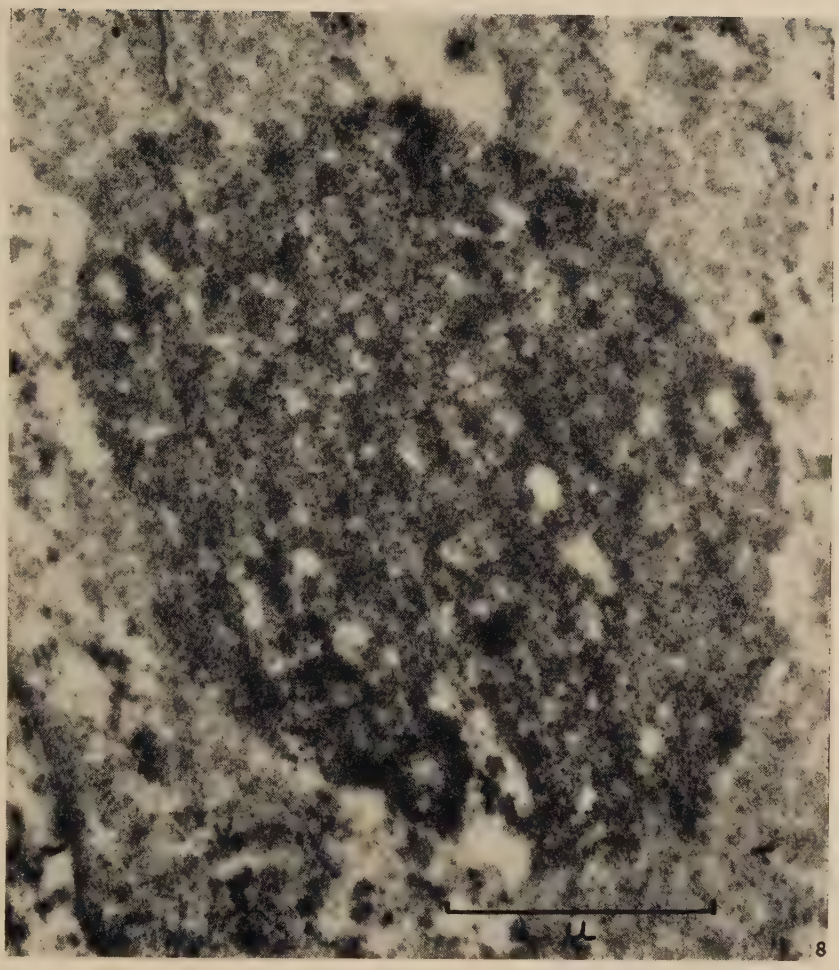


Fig. 8. *Nucléole d'une cellule d'hépatome.* Aspect réticulé très dense. Gross : 35 000 ×.

la cellule maligne est presque toujours très augmentée, sa structure fine reste la même. Elle a un aspect réticulé, avec ou sans masse homogène. Il y a des exceptions à cette règle : dans la tumeur d'Ehrlich, par exemple, la « pars amorpha » prédomine et souvent existe seule.

Ainsi nous n'avons pas trouvé de signes spécifiques et constants dans la structure du nucléole qui permette de distinguer une cellule maligne d'une cellule normale.

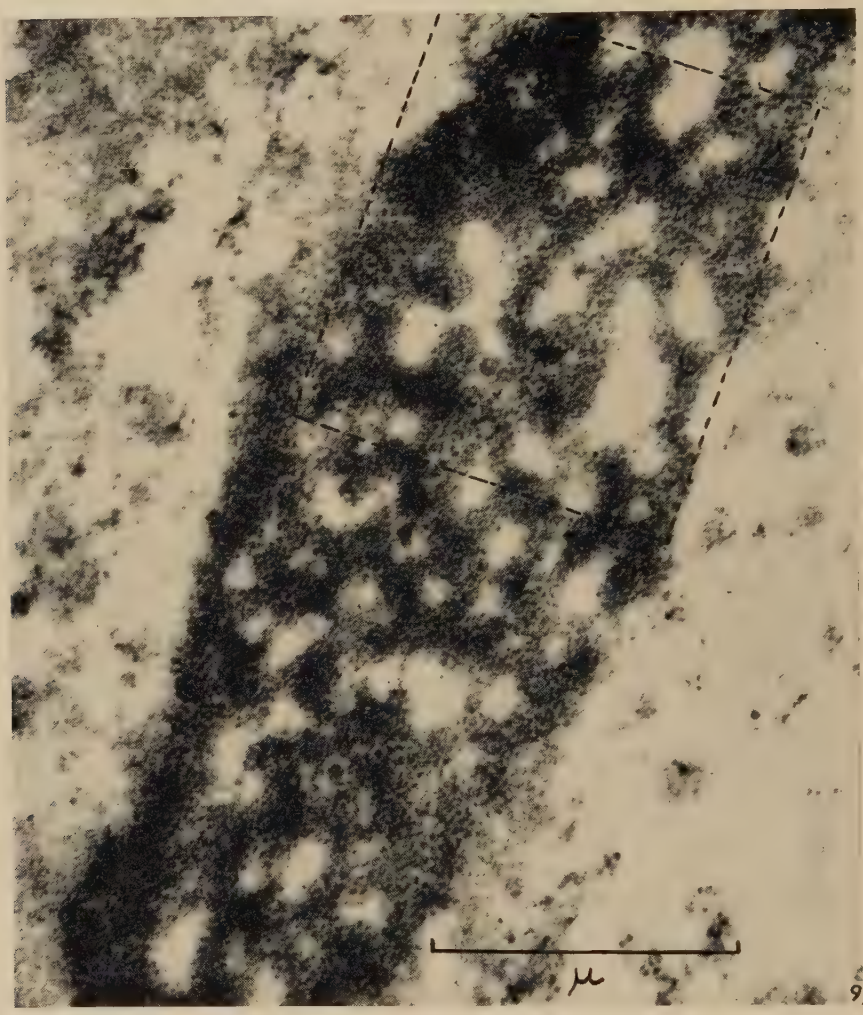


Fig. 9. *Nucléole d'un hépatome* formant un réseau plus ajouré que celui de la figure 8. Gross 40 000 ×.

DISCUSSION

a) Du point de vue *morphologique*, ces nouvelles études confirment les travaux d'Estable et Sotelo [9], de Borysko et Bang [3] ainsi que notre première note à ce sujet [1]. La plupart des cellules animales observées jusqu'ici provenant surtout de tissus de Vertébrés, présentent un nucléole *filamenteux* ou *réticulé* qui peut être enrobé dans une *masse diffuse* plus ou

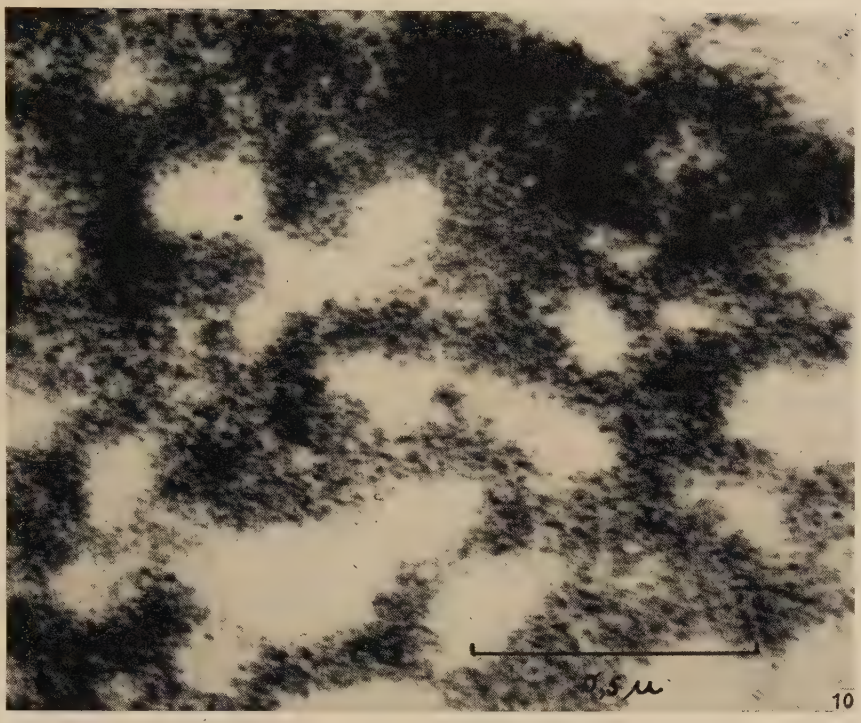


Fig. 10. Portion aggrandie de la figure précédente (correspondant au pointillé). Les grains se présentent comme des particules très denses, arrondies, parfois accolées en courts bâtonnets. Ils se détachent sur un fond plus clair formé par d'autres particules moins denses, plus allongées. Gross : 75 000 ×.

moins dense. Cette structure filamenteuse est facilement mise en évidence dans des cellules non fixées soumises à l'action d'une solution hypotonique [7]. Elle a été parfaitement démontrée par la microcinématographie dans des cellules vivantes [10, 5]. Tout récemment, les observations de Lettré et Lettré [11] en apportent de nouvelles preuves. L'existence réelle d'un organite filamenteux ne fait donc plus de doute. La terminologie proposée par Estable et Sotelo [9] pour désigner les deux constituants nucléolaires paraît tout à fait justifiée :

le *filament nucléolaire* deviendrait le « nucleolonema », la masse diffuse et homogène serait la « pars amorpha ».

Ces observations permettent en outre d'apporter une nouvelle contribution à la connaissance de l'ultrastructure nucléolaire : grâce à l'utilisation d'une meilleure technique, l'analyse structurale a pu être poussée jusqu'aux dimensions macromoléculaires, ce qui a révélé des composants gra-

nulaires ou filamenteux d'un ordre de grandeur de 100 Å dans le « nucleolonema » et la « pars amorpha ». Il ne s'agit bien entendu que d'un début d'investigation et l'interprétation des images de haute résolution doit être faite prudemment. Mais les images ainsi obtenues sont constantes et leur identification future à des constituants chimiques précis du nucléole ou du noyau reste possible.

b) Du point de vue de l'*histochimie actuelle*, le problème qui se pose est de savoir à quoi correspondent le « nucleolonema » et la « pars amorpha », et si l'on est en droit de les considérer comme de nature distincte. On sait que le nucléole est en général Feulgen-négatif et ne prend plus la pyronine après action de la ribonucléase, mais il est souvent entouré d'un anneau plus ou moins compact de substance Feulgen-positive qui résiste à l'action de l'enzyme.

Les colorations que nous avons effectuées sont en parfait accord avec les résultats de la plupart des chercheurs : Davidson [6], Yokoyama et Stowell [16] pour ne citer que deux des auteurs qui ont travaillé sur le même matériel.

Ces résultats correspondent aussi à ceux de Caspersson [4] qui propose le terme de « nucleolus-associated-chromatin » (NAC) pour désigner l'hétérochromatine qui souvent entoure d'un anneau le nucléole.

Comme nous l'avons déjà dit dans notre dernière publication, il nous paraît vraisemblable que le « nucleolonema » est porteur de l'acide ribonucléique. En effet, les nucléoles purement filamenteux apparaissent Feulgen-négatifs. Peut-on alors assimiler la « pars amorpha » intra et périnucléolaire à de l'hétérochromatine? Il est difficile de conclure car il est apparu au cours de ces recherches que l'emploi de fixateur autre que l'acide osmique entrainait la formation d'images artificiellement homogènes. En effet, au microscope électronique, une cellule hépatique fixée à l'acide osmique présente un nucléole purement filamenteux. L'action sur cette cellule de formol, par exemple, provoque l'apparition d'une pseudo « pars amorpha », qui peut prendre une disposition annulaire semblable à celle des images de la NAC observées au microscope électronique. Le fixateur de Carnoy, si souvent utilisé pour l'histologie courante du nucléole, réalise toujours au microscope optique une image nucléolaire permettant de distinguer une zone centrale et une zone périphérique nettement séparées l'une de l'autre, même dans les cas où l'acide osmique ne révèle qu'une structure filamenteuse. De telles zones pourraient donc correspondre à du matériel chromatinien condensé autour du nucléole lui-même. C'est pourquoi il est très audacieux de superposer les observa-

tions faites au microscope électronique à celles obtenues par les techniques d'histologie courante.

Quant à la *signification fonctionnelle* des parties filamenteuses et diffuses du nucléole, on ne peut qu'échafauder des hypothèses jusqu'à ce que l'identification biochimique des deux constituants soit faite. Comme nous l'avons vu, les grains contrastés et *arrondis* du « nucleolonema » pourraient représenter des complexes moléculaires de ribonucléoprotéines qui s'accolent à la membrane nucléaire, la traversent et deviennent les grains des structures ergastoplasmiques du cytoplasme. La théorie de Caspersson attribuant au nucléole un rôle dans l'élaboration des substances responsables de la basophilie cytoplasmique trouverait ainsi sa confirmation. Mais, si nos images ne s'opposent pas à cette interprétation, elles n'apportent pas non plus de preuves irréfutables. Même dans les cas où le « nucleolonema » reste collé à la face interne de la membrane nucléaire, celle-ci ne présente aucune discontinuité apparente à cet endroit, et si les grains traversent les membranes, nous n'avons pas pu obtenir d'images de leur passage. Mais ce problème devrait, en principe, être résolu dans un proche avenir par le microscope électronique. Pour le moment celui-ci ne peut que préciser la description de l'ultrastructure nucléolaire et ainsi contribuer à la mise en valeur d'un très petit, mais très complexe organite cellulaire dont l'importance n'échappe plus à personne.

RÉSUMÉ

L'étude au microscope électronique du nucléole de cellules normales et de cellules cancéreuses permet de préciser l'ultrastructure de cet organite.

L'existence d'un élément réticulé ou filamenteux (« nucleolonema ») et de zones intra et périnucléaires plus diffuses (« pars amorpha » selon Clemente Estable) est confirmée.

Grâce au développement récent de la technique des coupes ultrafines, l'ultrastructure de ces deux composants se révèle comme granulaire. Les dimensions des grains sont de l'ordre macromoléculaire. Leur identification aux constituants chimiques du nucléole est discutée.

Il n'a pas été constaté de différences ultrastructurales constantes entre les nucléoles des cellules normales et ceux des cellules cancéreuses.

SUMMARY

More information has been gathered about the structure of the nucleolus of normal and cancerous cells using the electron microscope.

The presence of a reticular or filamentous portion ("nucleolonema") and more diffuse intra- and perinuclear zones ("pars amorpha" of Clemente Estable) has been confirmed.

New methods of ultrathin sections have revealed that both have a granular ultrastructure. The dimensions of the grains being of macromolecular dimensions, their relation with the chemical constituents of the nucleolus is discussed.

No fundamental difference in the ultrastructure was found between the nucleolus of normal and cancerous cells.

Nous tenons à remercier la Mutuelle Générale de l'Éducation Nationale de son aide généreuse, Mlle E. LeBreton et Guérin qui nous ont fourni les tumeurs, ainsi que Madame Jacqueline Arnoult pour sa collaboration technique.

BIBLIOGRAPHIE

1. Bernhard, W., Haguenau, F., et Oberling, Ch., *Experientia* **8**, 58 (1952).
2. Bernhard, W., Gautier, A., et Rouiller, C., *Arch. Anat. Microscop. et Morphol. exptl.* **43**, 236 (1954).
3. Borysko, E. et Bang, F. B., *Bull. Johns Hopkins Hosp.* **89**, 468 (1951).
4. Caspersson, O., Cell Growth and Cell Function. W. W. Norton and C° Inc., N.Y., 1950.
5. Chèvremont, M. J. J., VIIIth Int. Congress for Cell Biology, Leyden, 1954.
6. Davidson, J. N., *Cold Spring Harbor Symposia Quant. Biol.* **12**, 50 (1947).
7. Denues, A. R. T., Communication personnelle.
8. Estable, C., *Congreso Médico del Centenario. Montevideo* **9**, 558 (1930).
9. Estable, C. et Sotelo, J., *Instituto de Investigaciones de Ciencas Biológicas* **1**, 105 (1951).
10. Frederic, J., *Bull. Assoc. Anat.* **72**, 111 (1953).
11. Lettre, R. A. et Lettre, H., VIIIth Int. Congress for Cell Biology, Leyden, 1954.
12. Palade, G. E., 11 Congrès Soc. Amer. Microscop. électr., Pocono Manor, 1953.
13. Sjöstrand, F. S. et Hanzon, V., *Exptl. Cell Research* **7**, 393 (1954).
14. Vigier, P., Le nucléole morphologie-physiologie. Thése Paris, 1900.
15. Vogt, C. et Vogt, O., *Ärztl. Forsch.* **1**, 8 (1947).
16. Yokoyama, H. O. et Stowell, R. E., *J. Natl. Cancer Inst.* **11**, 939 (1951).

SOME FACTORS IN NUCLEAR STIMULATION OF MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION *IN VITRO*

H. STERN[1] and S. TIMONEN[2]

Laboratories of the Rockefeller Institute for Medical Research, New York, U.S.A.

Received September 25, 1954

It is a curious fact that although terminal oxidation and its associated phosphorylation appear to be confined to mitochondria, a number of studies based on techniques as diverse as enucleation and cell fractionation should point to the possibility of the nucleus having a direct influence on the coupling of oxidation with phosphorylation in the intact cell. On the one hand, there are the highly suggestive results of Mazia and Hirshfield [5] and of Brachet [1] on the quick decline in the capacity of enucleated halves of *Amoeba* to incorporate inorganic phosphate from their medium even though their oxygen consumption remains undiminished. On the other, there are the *in vitro* experiments of Johnson and Ackerman [3] showing a marked increase in rate of oxidative phosphorylation upon addition of the nuclear fraction of liver homogenate to the mitochondrial suspension. The importance of such a relation—if it exists—is patent, but unfortunately, the studies supporting it are equivocal in their bearing. The significance of enucleation experiments to this question has been discussed by Brachet [2]; our results are addressed to the *in vitro* phenomena. Using livers of young rats, we have been able to confirm the results which Johnson and Ackerman obtained on fractionated embryonic chick livers. Our results, however, have also tended to the conclusion that the stimulation by nuclei observed *in vitro* has no counterpart *in vivo*, but arises from an artefact of *in vitro* metabolism.

METHODS

Livers from albino rats weighing about 80–120 gms were homogenized in 0.25 *M* sucrose and fractioned according to the method outlined by Schneider [7]. Mitochondria were suspended in a volume of sucrose giving a concentration equivalent

[1] Present address: Botany Department, Univ. of Pennsylvania, Philadelphia, U.S.A.

[2] Rockefeller Foundation Fellow. Present address: Department of Anatomy, Univ. Helsinki, Helsinki, Finland.

to that obtaining in a 40 per cent suspension of whole homogenate. The "nuclear fraction", which consisted of nuclei, whole cells, and assorted debris, was made up to a concentration equivalent to a 30 per cent suspension.

With the exception of hexokinase, all reagents were obtained from commercial sources. Hexokinase was prepared from yeast in partially purified form following the method of Kunitz and McDonald [4]. The amount of hexokinase added to each incubation mixture was sufficient to assure a non-limiting rate of conversion of glucose plus adenosinetriphosphate to glucose-6-phosphate. Oxygen consumption was measured by conventional manometric techniques. Ten minutes were allowed for equilibration and the reaction started by adding the tissue from one of the sidearms to the main well of the flask. The reaction was stopped with trichloracetic acid, and the deproteinized solution used for phosphorus analysis.

RESULTS

When rates of oxidation and phosphate esterification were followed against the concentration of mitochondria in the incubation medium, it was apparent that the activities per unit amount of mitochondria fell rapidly with dilution (Table I). Although this pattern of behaviour suggested the presence of an inhibitor in the medium, attempts to locate the apparent inhibition in one

TABLE I

Mitochondrial concentration in relation to oxidative activity.

Substrate	Mitochondria added (ml)	μatoms oxygen/ 10 minutes	μM phosphate esterified/ 10 minutes
Glutamate	0.4	3.9	9.7
	0.3	3.1	6.8
	0.2	1.35	3.3
	0.1	0.28	0.7
Succinate	0.1	4.2	—
	0.15	2.05	—

Mitochondria were added from a suspension containing 400 mg equivalents of whole liver/ml. For assays of oxidative phosphorylation the incubation medium contained the following components (expressed in μmoles): Adenosinetriphosphate, 3; Cytochrome C, 0.01; $MgCl_2$, 6; K glutamate, 30; dextrose, 54; phosphate, 15; hexokinase, 80–100 units (measured at 23°C); sucrose and water were added to give a final volume of 2.8 ml and an osmotic pressure of 0.8 isotonic. The pH of the mixture was 7.2. Incubation periods varied from 10–20 minutes. Succinoxidase activities were measured in the following medium: Sodium succinate (0.5 *M*), 0.3 ml; $CaCl_2$ (0.006 *M*), 0.2 ml; Cytochrome C (1.3 mg/cc in 0.25 *M* potassium phosphate buffer, pH = 7.2), 0.4 ml; sucrose (0.25 *M*), 1.05 ml; water and tissue to 2.8 ml. All activities were measured at a temperature of 30.5°C.

of the components of the reaction mixture were negative, as were tests for heavy metal contamination by use of the phenyl carbazone reagent. Terminal oxidation itself was unaffected since succinoxydase activity under similar conditions showed a proportionality at much lower concentrations of mitochondria. Thus, although the presence of some contaminating inhibitor could not be ruled out, it appeared to be unlikely so far as familiar sources of inhibition are concerned. Indeed, the failure of mitochondria to show proportionate activity with increasing dilution has been observed by other students of mitochondrial metabolism [8]. This *in vitro* characteristic of mitochondrial metobolism has an obvious bearing on the stimulating property reported for nuclei; from the standpoint of 'stimulation' alone, these results could be taken to mean that adding mitochondria to mitochondria was even more effective than adding to them an aliquot of a nuclear fraction.

The 0.2 ml of nuclear fraction used in the various experiments, if incubated by itself, showed relatively little oxygen uptake and a net loss of organic phosphate. Added to suspensions of mitochondria, however, this fraction served to stimulate both oxidation and phosphorylation to an extent that could not be accounted for additively. It was clear, therefore, that some elements of the nuclear fraction stimulated the mitochondria to higher rates of oxidation and phosphorylation. The pattern of this stimulation was, nevertheless, not simple. Results reported in Tables II–V compelled a number of qualifications:

(1) The potency of the stimulating effect was a function of mitochondrial concentration. Where only 40 mg equivalents of mitochondria were incubated there was a 4–7 fold increase in activity upon addition of the nuclear fraction; with 160 mg equivalents of mitochondria, the increase was in the order of 25 per cent. Thus the direction of the stimulation was towards a restoration of proportionality in mitochondrial activity (Table II).

(2) A nuclear fraction heated to 100° C for 1 minute was no less effective than the untreated fraction in stimulating the mitochondria. Actually, phosphate esterification was increased due to destruction of the adenosinetriphosphatase present in the nuclear fraction. Thus, in no sense could the observed stimulation be attributed to an *in vitro* metabolism of the nuclei; the effect in question could be due either to some physical property of the nuclear fraction or to some constituent heat stable metabolite, or to both. Centrifugation of the heated fraction for five minutes at 8500 g provided no sharp separation of stimulating property; in general, the resuspended residue was the more effective, but both residue and cloudy supernatant were 70–90 per cent as active as the undivided fraction. It seemed unlikely

therefore, that a soluble metabolite could be largely responsible for the stimulation of mitochondrial metabolism (Table III).

(3) At a given concentration of mitochondria, increase in the amount of nuclear fraction added was not paralleled by a corresponding increase in

TABLE II

Effect of "Nuclear Fraction" and serum albumin on oxidative phosphorylation.

Mitochondria incubated (ml)	Additions	μatoms oxygen	μM phosphate esterified
0.4	—	4.8	10
	NF	5.7	12.8
	Albumin	4.6	11.4
0.2	—	1.48	3.3
	NF	2.8	5.8
	Albumin	2.3	5.6
0.1	—	0.3	0.4
	NF	1.23	2.91
	Albumin	0.8	1.2

NF indicates Nuclear Fraction of which 0.2 ml was added. In the case of albumin, 12 mg of a crystalline preparation were added to the mixtures shown. All values are for 10 minute periods. The remainder of the conditions is the same as in Table I. In those cases where nuclei have been added, the values have been corrected for whatever metabolic activity was shown by the nuclear fraction incubated alone.

TABLE III

The effect of heating on the stimulating activity of the nuclear fraction.

Addition	μatoms oxygen	μmoles phosphate esterified
None	1.3	2.3
NF	2.3	3.9
NF, heated	2.2	5.5
None	3.1	7.4
NF, heated	4.9	12.3
NF, heated residue	4.0	10.2
NF, heated supernate	3.1	9.1

Conditions of incubation same as those in Table II. An aliquot of the nuclear fraction (NF) which had been heated for 1 minute at 100°C was centrifuged and the supernatant and residue (resuspended in sucrose) added in 0.2 ml volumes where indicated.

mitochondrial stimulation. Thus the amount of nuclear fraction supplied did not appear to be limiting; so that the greater effectiveness of the nuclear fraction at higher mitochondrial dilutions must have been due to the state of the mitochondria arising from the effect of dilution (Table IV).

(4) Serum albumin [6] stimulated different concentrations of mitochondria in a pattern similar to that of the nuclear fraction (Table II). The effects of these stimulants on the mitochondria were not identical since their combined addition resulted in stimulations which were equal to or exceeded the sums

TABLE IV

The oxygen consumption of mitochondria with variation in amount of heated nuclear fraction added.

Addition (ml)	μliters oxygen
None	10
0.1	21
0.2	26
0.3	19
0.4	23
0.5	21

Conditions of incubation were the same as in previous runs. 0.3 ml of mitochondria were used. The control activity was lower than in other runs but parallel assays with 0.1 ml of mitochondria showed the relative responses to addition of nuclei to be the same. Unheated nuclear fractions could not be used for this experiment, for it was found that the mitochondria present in the nuclear fraction show appreciable activity when volumes larger than 0.2 ml are added, an effect not unexpected in view of what has already been shown. Behaviour with respect to phosphate was the same as with respect to oxygen.

TABLE V

Comparison of increases in oxygen consumption due to the nuclear fraction and albumin administered singly or combined.

Mitochondria incubated (ml)	Albumin	NF	Albumin plus NF
0.1	1.5	15.5	23.5
0.2	7.5	19.5	29.5
0.3	6.0	20.0	25.0
0.4	5.0	13.0	21.0

Values recorded are for μliters oxygen consumed above that of the control without additions. Conditions of incubation were the same as indicated for previous runs.

of their individual stimulations. It remained unlikely, however, that a fundamental distinction could be made between the respective effects of the nuclear fraction and of serum albumin. If it were assumed that serum albumin opposed the destructive results of mitochondrial dilution and that the nuclear fraction supplied a metabolite which became limiting with progressive dilution of the mitochondria, then their combined effect should have exceeded the sum of their individual effects increasingly with mitochondrial dilution. It is clear from Table V that this was not so; if anything, the trend was in the opposite direction.

DISCUSSION

The results here presented attest to a complexity of the factors capable of stimulating *in vitro* mitochondrial metabolism. It would be difficult, under the circumstances, to select the particular case of the nuclear fraction and to draw definitive conclusions about physiological relations between nuclei and mitochondria. Such a procedure would pile error on error, for one would have to overlook not only the instability of the mitochondria under the conditions tested, but also the broad heterogeneity of the nuclear fraction. The single point of clarity in these experiments is that the nuclear fraction, like serum albumin and probably other substances, opposes the destructive effect of dilution on mitochondrial activity. The mechanism of this effect is beyond the scope of these results, but the probability is strong that at least part of it is in the nature of a physical effect on the condition of the mitochondria. Such an effect is a far cry from that required by the proposition that within the intact cell the nucleus exercises an immediate influence on the coupling of oxidation with phosphorylation. The influence may exist, but we do not consider any of the results of mixing nuclear fractions with mitochondria *in vitro* as relevant to the question.

Although the point would seem obvious that the substitution of a sucrose medium for the organized environment of the cell must expose mitochondria to a variety of untoward effects, it is quite often lightly taken. Any program of cell physiology seeking to resolve the interactions between subcellular components by *in vitro* combinations of fractions obtained from sucrose homogenates cannot assume that the fractions thus combined have a counterpart in the intact cell. One should consider the extent to which the whole homogenate or parts of it affect in a nonspecific way the integrity of the subcellular function studied where rate stimulation is the point in question. The observation of Johnson and Ackerman [3] that nuclei and mitochondria

centrifuged down together are more active than if isolated separately and then combined is understandable in light of some such nonspecific effect; it would be difficult to explain in terms of cooperating metabolic systems.

SUMMARY

The reported stimulation of mitochondrial oxidative phosphorylation by added nuclear fractions was investigated with livers of young rats.

In the presence of glutamate, but not in the presence of succinate, progressive dilution of mitochondria in the incubation medium is paralleled by a decline in their specific metabolic rate. The stimulating effect of the nuclear fraction functions to oppose the inactivating effect of dilution. Heated nuclear preparations are as effective as those maintained at 0° C up to the time of addition to the incubation mixture. The sum of the evidence suggests that the stimulation of mitochondria by nuclear fractions is due to some physical effect of such fractions on the mitochondria and does not represent a consequence of cooperating metabolic systems.

REFERENCES

1. Brachet, J., *Actualités Biochimiques* **16** (1952).
2. —— *Arch. Biol.* **65**, 1 (1954).
3. Johnson, R. B. and Ackerman, W. W., *J. Biol. Chem.* **200**, 263 (1953).
4. Kunitz, M. and McDonald, M. R., *J. Gen. Physiol.* **29**, 293 (1946).
5. Mazia, D. and Hirschfield, H. I., *Science* **112**, 297 (1950).
6. Saktor, B., *J. Gen. Physiol.* **37**, 343 (1954).
7. Schneider, W. C., Manometric Techniques and Tissue Metabolism. Burgess Publishing Co., Minneapolis, p. 148, 1949.
8. Siekewitz, P. (private communication).

A STUDY OF THE HORMONAL RELATIONS OF EPIDERMAL MITOTIC ACTIVITY *IN VITRO*

III. ADRENALIN

W. S. BULLOUGH

Birkbeck College, University of London, England

Received October 28, 1954

A STUDY of the effects of stress and shock *in vivo* has led to the conclusion that adrenalin, or possibly its oxidation product adrenochrome, has a powerful antimitotic action in adult mouse epidermis [3,] as it is also known to have in a number of other tissues [7, 8]. The present paper is an attempt to analyse more fully the relationship between adrenalin and epidermal mitotic activity in the critical conditions of a saline tissue culture medium.

MATERIAL AND METHODS

The ear epidermis of adult male mice of the Kreyberg WLL strain was used as the test object, the technique being that already described by Bullough [4]. The hormones were adrenalin chloride (Parke, Davis & Co. Ltd) and adrenalin tartrate (Burroughs Wellcome & Co.), and both were used in aqueous solution.

Adrenochrome, which was also tested, was prepared by neutralising and diluting an aqueous solution of adrenalin chloride, and by passing oxygen through it for 2 hr. at 25°C. At the end of that time the solution was bright red, and probably contained a mixture of several oxidation products, together perhaps with a small residue of unoxidised adrenalin. Adrenalin tartrate, being very stable, could not be broken down in this way.

OBSERVATIONS

Preliminary experiments were performed to test the action of adrenalin chloride on epidermal mitosis in the simple conditions of a phosphate-buffered saline medium. The results of two experiments involving the ear epidermis of a total of 10 adult male mice are given in Table I.

This shows clearly the antimitotic action of adrenalin, and demonstrates that in the conditions of the present experiments a complete inhibition is obtained with a concentration of about 10 γ per 4 ml culture medium. Thus adrenalin has the same effect *in vitro* as it is already known to have *in vivo*.

Adrenalin is known primarily as a substance which powerfully stimulates the phosphorylation of glycogen [5]. However a solution of adrenalin chloride, once it has been neutralised, is quickly oxidised to a series of coloured compounds known as the adrenochromes [1]. At least one of these is known to be a powerful inhibitor of the hexokinase and phosphohexokinase systems [9], and it is evident, since epidermal mitosis is dependent on active energy production, that the observed mitotic depression could be fully explained in terms of these inhibitions.

TABLE I

Average numbers of mitoses arrested by colchicine in 4 hr. in unit lengths (1 cm) of sections 7 μ thick of ear epidermis incubated at 38°C in 4 ml saline medium with 0.02 *M* glucose and an oxygen gas phase. Each figure is the average of 10 observations derived from two separate experiments.

Adrenalin chloride concentration per 4 ml culture medium				
0	5 γ	10 γ	50 γ	100 γ
9.6 ±0.80	1.7 ±0.61	0.2 ±0.16	0	0
Concentration of adrenalin chloride oxidised to adrenochrome per 4 ml				
0	5 γ	10 γ	50 γ	100 γ
10.2 ±0.66	0.4 ±0.12	0	0	0

The effects of adrenochrome, as the mixture of adrenalin oxidation products may for convenience be called, was therefore next determined, and the results are also given in Table I. A mitotic inhibition was again obtained, suggesting either that adrenalin and adrenochrome may both act as inhibitors in much the same manner, or that the inhibition exerted by adrenalin may in fact be due to the formation of adrenochrome in the culture medium.

An attempt to decide between these alternatives was made using adrenalin tartrate, which, being highly stable, does not readily oxidise to form adrenochrome. All attempts to oxidise it by neutralisation in the presence of oxygen failed, and in the conditions of the experiment no trace of the pink colouration of adrenochrome developed. The results of an experiment

involving 10 mice are given in Table II. Sodium tartrate in equivalent concentrations was used in the control.

From these results it is clear that, in the concentrations used, tartrate is not itself an inhibitor. It follows that the inhibition observed with adrenalin tartrate is most probably due to the adrenalin itself. Both adrenalin and adrenochrome appear therefore to exert a similar mitotic inhibition in the conditions of the present experiments.

TABLE II

Average numbers of mitoses arrested by colchicine in 4 hr in unit lengths (1 cm) of sections 7 μ thick of ear epidermis incubated at 38°C in 4 ml saline medium with 0.02 *M* glucose and an oxygen gas phase. Each figure is the average of 10 observations.

Concentration of adrenalin tartrate per 4 ml (weight of tartrate not included)				
0	5 γ	10 γ	50 γ	100 γ
9.9 ±0.62	1.0 ±0.18	0.5 ±0.17	0.4 ±0.13	0.3 ±0.12
Control with sodium tartrate in equivalent concentration				
9.9 ±0.62	9.4 ±0.27	11.1 ±0.86	9.7 ±0.44	10.0 ±0.71

An attempt was next made to define the mechanism of the inhibition. In the case of adrenochrome it could be anticipated from the results of Meyerhof and Randall [9] that the inhibition might be mainly due to a blockage of glucose uptake. To test the validity of this hypothesis fragments of ear, taken from 10 adult male mice, were incubated with glucose, fructose, or lactate as substrate, and the results are shown in Table III. The results of parallel experiments with adrenalin tartrate are also included in this table.

As before a mitotic inhibition was observed when either adrenalin or adrenochrome was added in the presence of glucose. In the presence of fructose or lactate the controls showed the higher rate of mitosis normally developed with these substrates [2], but in both cases the adrenalin-treated ears suffered a powerful mitotic inhibition. It follows from the lactate results that any inhibition to the glucokinase system or to the enzyme systems of glucolysis cannot be considered as the basis of the antimitotic effects of adrenalin or adrenochrome.

Still working on the assumption that adrenalin acts by inhibiting some phase of carbohydrate metabolism relating to energy production, the question of the tricarboxylic acid cycle was next examined, and the actions of adrenalin tartrate were determined in the presence of a number of the glucolytic and cycle intermediates. The main results obtained are given in Table IV.

TABLE III

Average numbers of mitoses arrested by colchicine in 4 hr. in unit lengths (1 cm) of sections 7 μ thick of ear epidermis incubated at 38°C in 4 ml saline medium with an oxygen gas phase. Each figure is the average of 10 observations.

Substrate 0.02 *M* glucose		Substrate 0.02 *M* fructose		Substrate 0.02 *M* *dl*-lactate	
Alone	10 γ adrenochrome	Alone	10 γ adrenochrome	Alone	10 γ adrenochrome
9.3 ±0.68	0.7 ±0.23	14.6 ±0.70	1.5 ±0.39	13.2 ±0.96	1.7 ±0.29
Alone	10 γ adrenalin tartrate	Alone	10 γ adrenalin tartrate	Alone	10 γ adrenalin tartrate
10.5 ±0.78	0.3 ±0.09	16.4 ±1.00	0.3 ±0.07	15.3 ±0.32	0.2 ±0.09

Two points emerge from these figures. The first is of general interest and concerns the fact that the further round the cycle a substance lies the poorer are its powers of supporting mitotic activity, a result which is in accordance with the suggestion that the further round the cycle the less energy remains to be extracted from the molecule.

The second point, which relates to the present argument, is that whenever significant mitotic activity is found adrenalin acts as a powerful inhibitor. It therefore appears improbable that the critical inhibition lies at any particular point in the tricarboxylic acid cycle, unless it be in one of later reactions, the stoppage of which might arrest the whole cycle.

Theoretically there remain the possibilities that the main blockage lies in the cytochrome system, the energy transfer system, or at some point outside the energy production pathways. As regards the cytochrome system two pieces of evidence are available. The first relates to an attempt to relieve any blockage to hydrogen transfer in the cytochrome system by the

addition of an alternative hydrogen acceptor. In these experiments methylene blue was used in an attempt to obtain some higher level of mitotic activity in the presence of adrenalin, but no success was achieved.

The second piece of evidence was obtained from the results of determinations of the rate of oxygen uptake of mouse ear epidermis and of mouse and rat diaphragm. The details of these experiments, which were carried

TABLE IV

Average numbers of mitoses arrested by colchicine in 4 hr. in unit lengths (1 cm) of sections 7 μ thick of ear epidermis incubated at 38°C in 4 ml saline medium with an oxygen gas phase. Each figure is the average of 10 observations.

Substrate					
0.02 *M* glucose		0.02 *M* fructose		0.02 *M* lactate	
Alone	10 γ adrenalin tartrate	Alone	10 γ adrenalin tartrate	Alone	10 γ adrenalin tartrate
10.9 ±0.82	0.2 ±0.05	16.4 ±1.28	0.1 ±0.04	16.0 ±1.04	0.2 ±0.06
0.02 *M* pyruvate		0.02 *M* citrate		0.02 *M* α-ketoglutarate	
Alone	10 γ adrenalin tartrate	Alone	10 γ adrenalin tartrate	Alone	10 γ adrenalin tartrate
12.6 ±0.85	0.1 ±0.05	4.5 ±0.45	0	4.2 ±0.43	0.1 ±0.03
0.02 *M* succinate		0.02 *M* fumarate		0.02 *M* malate	
Alone	10 γ adrenalin tartrate	Alone	10 γ adrenalin tartrate	Alone	10 γ adrenalin tartrate
4.2 ±0.42	0.1 ±0.03	0.4 ±0.01	0	0.2 ±0.06	0

out by means of the normal Warburg technique, need not be repeated here, but the results in all cases indicated that neither adrenalin nor adrenochrome have any statistically significant effect on respiration as measured in terms of oxygen uptake. This is in agreement with the results of E. Walaas [10] who used rat diaphragm, and it confirms the absence of any marked

inhibition in either the cytochrome system or the tricarboxylic acid cycle. There remain the possibilities of blockages in the phosphate transfer system or at some point outside carbohydrate metabolism altogether. These possibilities are discussed below.

DISCUSSION

In previous publications stress has been laid on the critical importance to epidermal mitosis of a high rate of energy production through the Krebs tricarboxylic acid cycle [2]. Any inhibition of glucolysis, the Krebs cycle, the cytochrome system, or the energy transfer system results in an immediate mitotic inhibition at some point prior to the prophase. This type of mitotic inhibition in the "pre-prophase", better called the antephase, has been contrasted with that induced in the metaphase by so many "mitotic poisons", and it has been suggested that an antephase inhibition may be taken as a sign that the inhibitor in question is interfering at some point in the process of energy production.

It has further been tentatively suggested [3] that the mitotic inhibition induced by adrenalin in mouse epidermis may in fact be due to an oxidation of adrenalin to adrenochrome, which is described as an inhibitor of hexokinase and of phosphohexokinase [1]. From the present evidence this view must now be abandoned and it must be admitted that adrenalin is an inhibitor in its own right. The question of whether either adrenochrome or adrenalin acts through some interference in the course of energy production is also now in doubt.

The addition of adrenalin to diaphragm muscle *in vivo* and *in vitro* results in a more rapid breakdown of glycogen, an accumulation of glucose-6-phosphate, and an increased production of lactic acid [11]. This is commonly taken to indicate an increased rate of energy output, if only for a minute or two, although the point does not yet seem to have been established.

Apart from this evidence adrenalin has been considered mainly as an inhibitor. It is described as reducing the rate of glucose uptake (11), and it is now well known that it inhibits mitotic activity in cultures of chick fibroblasts [7, 8] just as it does in mouse epidermis.

While the reduction in the rate of glucose uptake could be the basis of both these mitotic inhibitions, the present evidence indicates that this explanation is not adequate. If a glucokinase inhibition exists at all in epidermis, it is clearly overshadowed by a further inhibition at another point

in cell metabolism. This further inhibition does not appear to lie in the course of glucolysis, the Krebs cycle, or the cytochrome system, but it may possibly exist somewhere in the phosphate transfer mechanism in the manner of the well-known 2,4-dinitrophenol effect. This possibility cannot be tested by the present technique, but it has been considered by E. Walaas [10] in relation to rat diaphragm muscle and she concludes that "apparently the mechanisms of the effects of epinephrine and of 2,4-DNP on carbohydrate metabolism of the diaphragm are quite different, although the final changes are similar".

Incidentally, if adrenalin does indeed inhibit some step in the energy transfer system, it follows that the observed stimulus to glycolysis cannot result in the production of extra available energy, so that the basic function of adrenalin becomes even more obscure.

While some inhibition to phosphate transfer cannot yet be ruled out, the further possibility must be examined that the anti-mitotic action of adrenalin may be due to some critical inhibition in protein or nucleic acid metabolism, similar to that already suspected in fat metabolism [6]. If true this would suggest that a mitotic inhibition operating in the antephase may be due to causes other than those involving carbohydrate metabolism, and the question is now being investigated.

SUMMARY

1. An examination has been made of the theory that the antimitotic activity of adrenalin may be due to an inhibition at some point in carbohydrate metabolism, caused either by the adrenalin itself or by its oxidation product adrenochrome.

2. The results indicate first that adrenalin is an inhibitor in its own right, and second that, whether or not secondary blockages are created in the main pathways of energy production, the critical blockage is not to be found in glucolysis, the Krebs cycle, or the cytochrome system.

3. The unexplored possibilities remain that the main antimitotic action of adrenalin may lie either in some interference in the mechanism of energy transfer or in an inhibition of some critical reaction in protein or nucleic acid metabolism.

I wish to express my gratitude to the British Empire Cancer Campaign and to Organon Laboratories Ltd. for generous grants towards the expenditure connected with this work. Part of the expenses were also defrayed by the Central Research Fund of the University of London.

REFERENCES

1. Bacq, Z. M., *J. Pharmacol.* **95**, suppl. 1 (1949).
2. Bullough, W. S., *Biol. Rev.* **27**, 133 (1952).
3. —— *J. Endocrinol.* **8**, 265 (1952).
4. —— *Exptl. Cell Research* **7**, 176 (1954).
5. Cori, C. F., *Rep. 1st Inter. Congr. Biochem. Cambridge* (1950).
6. Haugaard, E. S. and Stadie, W. C., *J. Biol. Chem.* **200**, 753 (1953).
7. Lettré, H., *Z. physiol. Chem.* **271**, 200 (1941).
8. —— *Naturwissenschaften* **30**, 34 (1942).
9. Meyerhof, O. and Randall, L. O., *Arch. Biochem.* **17**, 171 (1948).
10. Walaas, E., Thesis submitted to the University of Oslo (1954).
11. Walaas, O. and Walaas, E., *J. Biol. Chem.*, **187**, 769 (1950).

THE GRADIENT TISSUE CULTURE METHOD[1,2]

E. E. OSGOOD and M. L. KRIPPAEHNE

Division of Experimental Medicine, University of Oregon Medical School, Portland, Ore., U.S.A.

Received November 1, 1954

A SIMPLE method of tissue culture is described which provides a continuous gradient in cell number, oxygen tension, oxidation-reduction potential and cell conditioning factors. The importance of oxygen tension [2, 4, 9, 24], oxidation-reduction potential [13], and conditioning [5, 6] of the medium by cells in tissue culture has been pointed out repeatedly. Various more [2, 3, 20, 21] or less [8, 13, 17, 22] complex devices have been designed to control these factors. Yet no method described in the literature appears to give as simple and quantitative control of these factors as the method presented herein and still permit continuous, not stepwise, variation.

During 18 years of culturing cells from human leukemic blood and blood-forming organs,[3] many variations in composition of medium, depth of layer, and oxygen tension have been investigated [18], none of which made any very significant improvement over the simplest techniques employed, until this simple method was developed. This has consistently given with any material so far cultured results far superior to those previously obtained. It seems worth while, therefore, even after only preliminary studies with the method, to make it available to others.

In brief, the method consists of introducing a glass slide at an approximately 45° angle into a wide mouth French square pint bottle containing 125–300 ml of suspension type culture (Fig. 1). The result is a continuous gradient in number of cells from top to bottom of the slide with a continuous gradient of oxygen tension and oxidation-reduction potential, and an opportunity for mature cells to migrate over the edge of the slide and drop to the bottom.

---

[1] This work was supported in part by a cancer control grant from the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service, and a grant from the U.S. Atomic Energy Commission.

[2] Presented before the Tissue Culture Association at Galveston, Texas, April 6, 1954.

[3] Terms and definitions used in this article conform to the recommendations of the Committee for Clarification of the Nomenclature of Cells and Diseases of the Blood and Blood-forming Organs. *Am. J. Clin. Path.* **18**, 443–450 (1948); **19**, 56–60 (1949); **20**, 562–579 (1950).

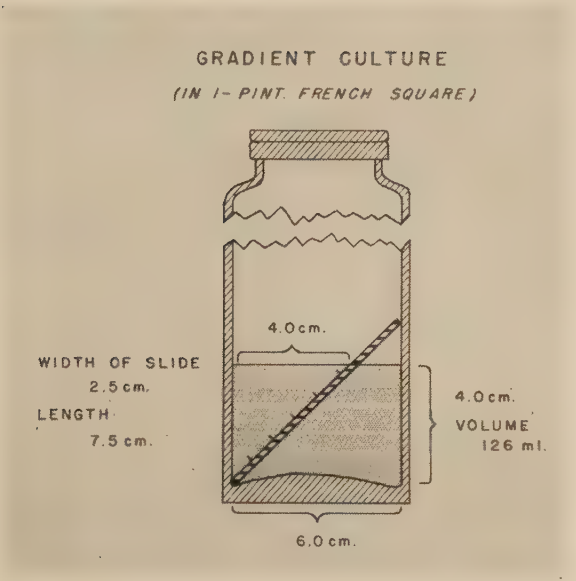


Fig. 1.—For most cell types a volume of 170 ml with a depth of 5 cm is preferable.

METHOD

The bottles used are screw cap wide mouth French square one pint bottles.[1] The screw caps are siliconized[2] after thorough cleaning, and care is taken in all manipulations not to allow the medium to contact the caps. The slides are standard new 1 × 3 inch glass slides. All manipulations are with syringe and needles of assorted sizes. Long spinal puncture needles are essential for aspiration of the mixed culture. Glassware is cleaned and sterilized by standard tissue culture techniques, the bottles being autoclaved with the caps unscrewed but in place. The cap and mouth of the bottle are flamed by standard bacteriologic technique whenever the bottles are opened. The slide is introduced and removed with long sterile tissue or sponge forceps.

The medium used in most of the cultures reported herein consists of $^1/_3$ reconstituted, pooled, lyophilized human serum[3] readjusted to pH 7.4 with CO_2 and $^2/_3$ balanced salt solution of the composition listed in Table I, to which 100 units of potassium penicillin G/ml are added. Any of the widely used fluid tissue culture media may, however, be as good or better. The gas phase in the studies to date is room air.

The number of cells inoculated has varied from 100 to 4000 per cmm, but 1000 per cmm seems to be a good number to aim at in initial studies with the method. Obviously, the greater the number of cells inoculated the higher on the slide cell growth will occur and the more often pH adjustment and medium changes will be necessary. The source of material has been aspirated blood or marrow from leukemic or nonleukemic individuals, or tissue cultures of previously isolated carcinoma cells. It may be used

[1] Manufactured by the Dura Glass Company, Owens, Illinois.
[2] Dri-Film 9987 (General Electric) was used for this purpose.
[3] Obtained from Hyland Laboratories, 4534 Sunset Boulevard, Los Angeles 27, Calif.

for lymph nodes, spleen, or tumor that has been passed through a tissue press. Mature erythrocytes are eliminated by the phytoagglutinin technique [16]; and the anticoagulant, usually heparin, although sodium citrate is equally satisfactory, is washed from the cells by centrifugating three times from the balanced salt solution, taking care never to pack the cells.

TABLE I

Modified balanced salt solution.

Components	Gm/l
$NaCl$	7.64
KCl	0.37
$CaCl_2$	0.224
$MgCl_2 \cdot 6H_2O$	0.20
Na_2HPO_4 (anhydrous)	0.12
KH_2PO_4	0.03
$MgSO_4 \cdot H_2O$	0.07
CH_3COONa	0.50
Dextrose	4.00
Distilled water to make 1000 ml.	

Enough medium is prepared from the stock of balanced salt solution and fresh reconstituted, lyophilized human serum to give the desired volume in the culture flask and to allow for one or two 25–50 per cent changes of medium. The volume necessary to give the desired depth is introduced into the sterile French square bottle with 50 or 100 ml syringes. Approximately 34 ml = 1 cm of depth, and 4 to 8 cm of depth will bring the level to the optimal range on the slide for most cell types so far studied. One hundred seventy ml will just reach the top of the slide at a depth of about 5.2 cm (5.0 cm above the lower edge of slide). Enough cells in concentrated suspension to give the number desired in the initial culture, as determined from hemocytometer counts, are introduced and the culture is throughly mixed by clockwise and counter clockwise rotations, with the bottom of the bottle resting on a flat surface. A sterile glass slide from the stock kept in petri dishes is then carefully introduced and adjusted in central position and the bottle placed in the incubator at 37°C.

At any desired interval the slide may be carefully removed with forceps[1], set on its side to air dry, and stained by any desired method, usually Wright's stain, or a cover slip may be placed over the slide, sealed and warm stage microscopic observations made. Wet fixation is obviously also possible. After removal of the slide, the culture is again mixed, and 2 ml of culture are removed for cell counts and smears for differential cell counting. Each 2 ml sample is subdivided into two aliquots, one measuring

[1] If desired, the loosely adherent cells may be first removed from the slide by aspirating cell-free medium from the top of the culture and washing the slide with this medium as it is withdrawn. After it is withdrawn it may be washed again with a stream of balanced salt solution. This will give better detail and show, of course, only those cells that are very firmly adherent to the glass surface. However, none of the figures were made from slides prepared in this way.

1.5 ml and the other 0.5 ml, which are placed in 5 × 50 mm test tubes. Leukocyte counting pipettes filled to the 1 mark with 30 per cent acetic acid with methylene blue are filled to the 11 mark for the total nucleated cell counts from the 0.5 ml tubes. The 1.5 ml tubes are centrifugated and the supernatant fluid is decanted for glass electrode pH determination. The sedimented cells are resuspended in the small amount of fluid remaining in the test tubes, and thin smears made and stained with Wright's stain for differential cell counting. At least 400 cells are counted in each total nucleated cell count and 500 cells in each differential cell count.

In many of the preliminary cultures reported herein, 20 ml of mixed culture were removed from the bottle as controls before introduction of the glass slide. These control samples were introduced into 100 ml vaccine vials and cultured by our standard technique [19] at a depth of about 8 mm.[1] These showed far lower counts after the first few days than in the corresponding gradient cultures.

The pH is adjusted as necessary to maintain it between 7.2 and 7.6 by adding CO_2 gas or allowing CO_2 gas to escape from the culture by loosening the screw cap. As a rule, lymphocytic or plasmocytic cultures become more alkaline, and all other cultures so far tested become more acid.

Medium changes are made at a time when the cells are completely settled out by withdrawing slowly and carefully one-fourth to one-half the volume and replacing it with an equal volume of the same medium initially used. Optimal frequency varies with the cell type and cell number, but is required less often than pH adjustment. If desired, the slide may be transferred aseptically to a new bottle containing one-half to one-fourth cell free conditioned medium from an earlier culture and the balance new medium without disturbing the cells on the surface. After each sampling, a volume of medium equal to that withdrawn is replaced.

The number of cells removed on a slide may be calculated by counting with the Miller disk [1] the number of cells in a central longitudinal strip the entire length of the slide and multiplying by the ratio of the width of this strip to the width of the slide, 2.54 cm. The horizontal distribution of cells is very uniform except at the extreme edges, but the vertical distribution is extremely variable, depending on the cell type and number. The number of cells on the slide is always much lower than the number calculated to settle on the slide[2] and of widely different proportions of cell types.

[1] M. L. Krippaehne and E. E. Osgood, *Acta Haematol.* **13**, 145 (1955).

[2] In calculating the number of cells which should settle on the slide it is convenient to diagram to exact scale on mm paper the diagonal plane of the upper surface of the slide and the inner face of the bottle, using the left-hand scale for depth and the right-hand scale for volume with a strip of mm paper below and parallel to the slide surface, so that measurements from the deep end along the slide may be identified. The exact coordinates for the slide surface are bottom at 0, top 50 mm above and 57 mm to right, length 7.62 mm, angle between 41 and 42°. One cm depth = 34 ml. A horizontal section of the bottle is a 6 cm square with diagonals joining points 1 cm from each corner.

It is also convenient to plot on log-log paper the volume directly above the slide on the ordinant and 2 scales on the abscissa, cm from bottom edge of the slide along its surface and the corresponding total volumes in the flask. On another sheet of log-log paper plot per cent of volume over slide on ordinant and same data on abscissa. The coordinants for these two straight lines are: for the abscissa top of slide = 7.62 mm or 170 ml and 2 cm = 44.6 ml. The y coordinants are 2 cm = 2.5 ml or 5.6 per cent, 7.62 cm = 36.2 ml or 21.3 per cent. For greater volumes add to 36.2 ml, 42.6 per cent of the difference between the total volume and 170 ml. Each 34 ml of culture equals 1 cm of depth and, above 170 ml, 14.48 ml over the slide.

DISCUSSION

It is obvious that the oxygen tension, oxidation-reduction potential and conditioning of the medium are all functions of the number and metabolic pattern of cells/unit volume of medium, the pressure and composition of the ambient gas mixture, the depth below the gas liquid interface, and the diffusion rate in the intervening liquid or solid phases. The method described, while extremely simple, permits of quantitative repetition and control of any of these factors and gives a continuous, not stepwise, gradient in each of these factors, and probably also in pH and CO_2 tension. The results obtained with the method are illustrated in Fig. 2–14.[1] For each type of cell there seems to be an optimal depth for a particular initial concentration of cells/unit volume of medium. Of the cell types studied the order of preferred oxygen tension from the highest to the lowest was bronchogenic carcinoma, plasmocytic myeloma, the neutrophilic granulocytic series, the eosinophilic granulocytic series, the monocytic series, the lymphocytic series, and the erythrocytic series. The range of preferred depth, with room air as the gas phase and about 1000 cells per cmm in the culture, was from a few mm to over 5 cm, although none of the hemic cells did well at depths less than 1 cm.

Since both cell count and depth influence the gradient, a rough guide to optimum level is the product of cell count in thousands per cmm and depth in cm of best growth. This product is called the gradient factor. This will equal best growth depth in a culture containing 1000 cells per cmm (Table II). For example, in a culture with a 3500 per cmm nucleated cell count, progranulocytes will be most numerous at a depth of 1 cm; with a 1000 count, at 3.5 cm; and with a 500 count, at 6 or 7 cm.

In most cultures the cells at optimum depths showed absolute increases in number and in some cultures the total cell counts showed significant increases even without correction for cells removed.

Culture K88A contained initially 4×10^5 cells of the plasmocytic series and by 8 days even after removal of three 2 ml samples and three gradient slides there were 5 times as many or 20×10^5 plasmocytic cells in the culture. Large areas of the gradient slides showed almost 100 per cent cells of the plasmocytic series.

Culture J92A increased from 1400 total nucleated cell count on day 1 to 2000 total nucleated cell count on day 4, although 3 samples and slides were

---

[1] We are indebted to John Brooke, cytologist and cytogeneticist in the Division of Experimental Medicine, for the photomicrographs and the J cultures.

removed. Contrast this with a drop by 8 days to 20 per cent of the initial count in the 8–12 mm deep standard cultures.[1]

Culture K87A showed an increase in total eosinophils from 1.5×10^6 at 0 time to 2.3×10^6 at 5 days although 3 slides and 3 2-ml samples were removed. On some of these gradient slides many fields were observed in which the cells were exclusively eosinophils in all stages of differentiation.

TABLE II

Peak and range of gradient factors for best cell growth.

Cell type	Gradient factor*	
	Peak	Range
Bronchogenic carcinoma	0.4	0.3 to 0.5
Plasmocytic series	1.5	0.5 to 2.5
Granulocytic series		
Neutrophils	3.5	1.5 to 6.0
Eosinophils	4.0	0.5 to 8.0
Monocytic series	4.5	1.0 to 8.0
Lymphocytic series.	6.0	2.0 to 10.0
Erythrocytic series	8.0	3.5 to 12.0

* Depth in cm. × cell count in thousands/cmm. Ranges in individual cultures are narrower.

Leukocytes from the blood of acute leukemias, which disintegrate very promptly in shallower cultures, grew well (see Figs. 2–8); each type of cell grew in a pattern similar to that observed in the hematopoietic organs, even though all were started from cell suspensions that had never been centrifugated tightly together, so that they must have settled on the slide in a random pattern of isolated cells. Promonocytes from acute monocytic leukemic blood produced cells closely resembling fibroblasts (Fig. 5), tissue histiocytes, and various giant cells, yet smears from the shaken cultures aside from showing the giant cells, showed the typical morphology of leukemic promonocytes (Figs. 6 and 7) with nothing resembling a fibroblast. In cultures containing promonocytes, whether from blood or marrow, giant cells resembling Langhans' giant cells were seen. The gradient factor for the giant cells apparently derived from the monocytic series was usually about 4.0–5.0. Such developments from promonocytes have been previously

[1] See footnote 2, p. 119.

described and illustrated [8, 12, 15, 23]. Note that at the optimal level for each cell type, the cells far overgrow other types present in the same culture so that it seems probable that this will prove an excellent method for isolating pure cell types from a mixed culture. As illustrated in Fig. 2, it was uniformly observed that even at optimal depth the number of cells near the edge of the slide is decreased in a narrow band. It seems probable that this may be a

---

In Figs. 2–14 the magnification is indicated by the scales on the figures. All were from cultures of adult human source and all were stained with Wright's stain using buffer phosphate of pH 6.4. The gradient factor is the product of the total nucleated cell count in thousands/cmm when the slide was introduced and the depth of the field shown in cm below the surface of the culture, and these numbers are given in parentheses after it in that order.

Figs. 2A and 2B. —Strip from edge of gradient slide from culture $K75A_6$ from the blood of acute leukemic lymphocytic leukemia to show pattern of distribution of cell growth on the slide. Retouched line marks top of fluid on slide. Slide introduced on day 3 and removed on day 6. Volume 126 ml, maximum depth 37 mm. Gradient factor 0 3.7 (1.0, 0.0–3.7). Shallow end at 2A left and deep end at 2B right. Note sparse growth at shallow depth, then a few colonies microscopically identified as belonging to the granulocytic and monocytic series; the majority of this slide is covered with colonies of prolymphocytes and lymphoblasts (Fig. 3), with peak density towards the bottom. Microscopic study shows a few colonies of nucleated erythrocytes interspersed at the extreme bottom. Due to the myelophthisic anemia progranulocytes and nucleated erythrocytes were present in the original blood.

Fig. 3.—Same as 2 at higher magnification to show pattern of growth of prolymphocytes and lymphoblasts which somewhat resembles follicles of lymphatic tissue. Note typical "tennis racket" pseudopods of isolated cells characteristic of lymphocytic series when fixed while motile. Gradient factor of this field 2.5 (1.0, 2.5).

Fig. 4.—Gradient slide from culture $K75A_{31-10}$ from blood of patient with chronic lymphocytic leukemia to show pattern of growth of more mature cells of lymphocytic series. This blood was added to mixture of pre-conditioned medium from previous culture of acute lymphocytic leukemia and fresh medium at 21 days. Gradient slide introduced at 8 days and removed at 10 days. Note tendency of cells to grow in cords as in lymph nodes. Volume of culture 160 ml. Gradient factor for this field 4.5 (1.5, 3.1).

Fig. 5.—Gradient slide from culture $K74A_9$ from blood of acute leukemic monocytic leukemia to show pattern of growth of promonocytes and their resemblance to fibroblasts. Slide introduced on day 6 and removed on day 9. Volume of culture 128 ml. Gradient factor for this field 3.9 (1.6, 2.4).

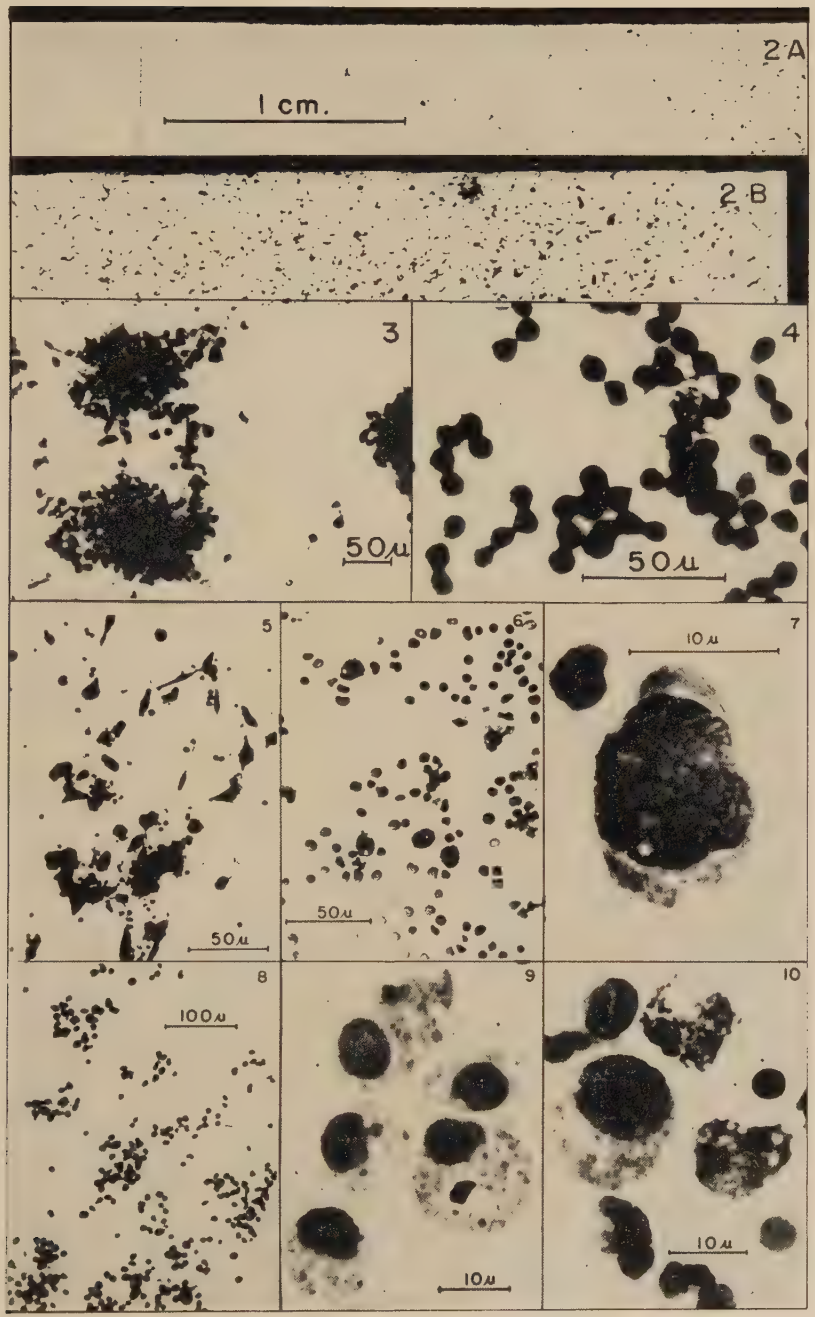
Fig. 6.—Smear from mixed culture made immediately after removal of gradient slide of Fig. 5 to show that in this type of preparation the fibroblast-like cells have the typical morphology of the promonocytes of blood. Contrast the abundance of mature erythrocytes with their sparsity in 5.

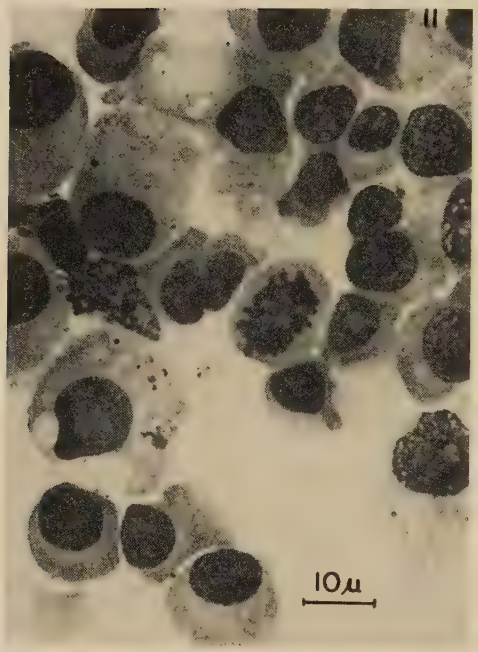
Fig. 7.—Same as 6 at higher magnification. Note typical promonocyte morphology.

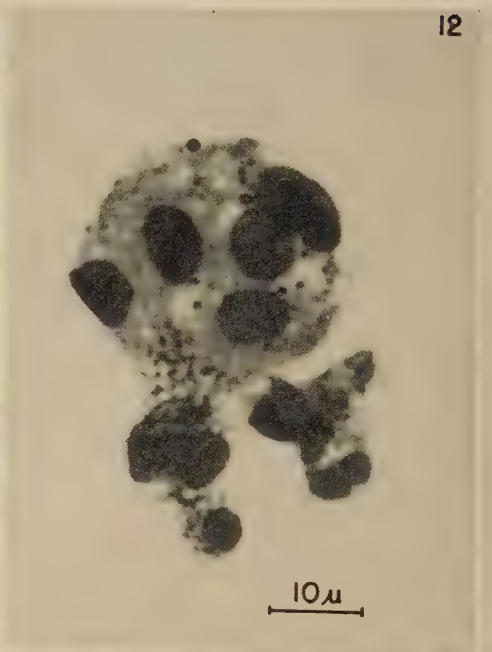
Fig. 8.—Gradient slide from culture $K77A_3$ from blood of acute terminal phase of chronic leukemic granulocytic leukemia to show pattern of growth of granulocytic series, chiefly in loose sheets of cells. Slide introduced on day 1 and removed on day 3. Note overlap with monocytic and lymphocytic series at this gradient factor. Volume of culture 130 ml. Gradient factor of this field 5.8 (2.9, 2.0).

Fig. 9.—Gradient slide from culture $K87A_{10}$ from marrow of patient with infectious arthritis to show pattern of growth of progranulocytes and early (nucleoli present) neutrophilic myelocytes. Note flat sheet and that more mature neutrophils have migrated away. Slide introduced on day 7 and removed on day 10. Volume 150 ml. Gradient factor for this field 1.0 (0.3, 3.5).

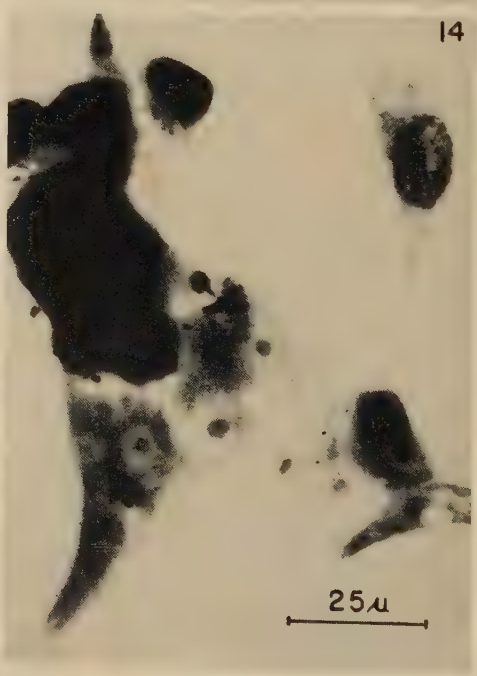
Fig. 10.—Smear from mixed culture made immediately after removal of gradient slide of Fig. 9 to show mixed marrow cell population including segmented eosinophil, band neutrophil, early neutrophil myelocyte and mature and nucleated erythrocytes.


2A
1 cm.
2B
3
50μ
4
50μ
5
50μ
6
50μ
7
10μ
8
100μ
9
10μ
10
10μ


11
10μ


12
10μ

13
10μ


14
25μ

conditioning effect due to the fact that the cells at the edge are not surrounded by cells on all sides.

Cultures identical in source, cell count and medium, at the depth that appeared to be ideal, but containing no gradient slide, were far superior to our previous cultures made at depths of 8–12 mm, but were definitely inferior in cell count to the corresponding gradient cultures. The most probable explanation for this is that the mature cell of the series produces an inhibitor of mitosis and that the mature cells, being more motile, migrate to the edge of the slide and drop off. Mature cells from each series were always found in the stained smears of the shaken culture after removal of the slide, except, of course, in the cultures of the acute leukemic cells, while they were sparse or absent on the gradient slide at the levels of optimal growth. Another reason why cultures of similar depth were not as successful is undoubtedly that the optimal level does change with time, especially in the early period in the gradient culture. After the culture is shaken and a new slide inserted the oxygen tension will at first be uniform and higher than at equilibrium, and it seems probable that the depth for optimal growth may be greater early in the experiment and gradually creep up the slide as equilibrium occurs. Furthermore, a wide range of biologic variation has been noted in every cell population and characteristic so far studied, and it seems probable that individual cells of a population may have individual preferences extending

---

Fig. 11.—Gradient slide from culture $K88A_6$ from marrow of patient with plasmocytic myeloma to show growth pattern of plasmocytic series. Slide introduced on day 2 and removed on day 6. Note tendency to grow in sheets, and the anaphase and prophases of mitosis. Volume of culture 123 ml. Gradient factor for this field 0.8 (0.37, 2.3).

Fig. 12.—Gradient slide from $K88A_{14}$ from marrow of patient with plasmocytic myeloma to show multinucleated giant proplasmocyte and segmented eosinophil has pseudopod extending across it. Slide introduced on day 12 and removed on day 14. Gradient factor for this field 0.6 (0.18, 3.3).

Fig. 13.—Gradient slide from culture $J92A_1$ from marrow of a different patient with plasmocytic myeloma to show pattern of growth of nucleated cells of the erythrocytic series. Slide introduced on day 0 and removed on day 1. Volume 200 ml. Gradient factor for this field 11.5 (3.5, 3.3). Note relative absence of mature erythrocytes on gradient slides. These were always found in the mixed culture after removal of the gradient slide. Note the progressive increase in maturity of the cells from the prorubricyte at the bottom of the group through rubricytes to the metarubricyte at the top of the group. Each cell from below upwards is slightly more mature in morphology than the adjacent cell.

Fig. 14. -Gradient slide from culture $K89A_2$ subculture from a tissue culture of bronchogenic carcinoma from pleural fluid of a patient named Maben first isolated in April, 1953, and maintained until February, 1954, by Dr. Arthur Frisch of the Department of Bacteriology of the University of Oregon Medical School in shallow culture. Volume of culture 170 ml. Gradient factor 0.3 (0.7, 0.5). Not only were there no cells found at a depth greater than 0.5 cm on the gradient slide there was a count of 0 on the mixed culture after removing the gradient slide showing that all cells introduced except those at very shallow depths had undergone complete autolysis in these 48 hours.

over a moderate range. Cultures in the same wide mouth French pint bottles that were tilted to one side at a similar angle, but contained no gradient slide, were also inferior to the gradient culture, possibly because the mature cells could not migrate over an edge.

These observations perhaps explain the tendency of ectopic bone marrow to develop in areas of relative anoxia and the fact that chronic exposure to 100 per cent oxygen at ordinary barometric pressures is toxic to man [14]. Perhaps metabolic studies should be repeated at optimal oxygen tensions for cell growth rather than in the 95 per cent oxygen 5 per cent CO_2 mixtures commonly used. Certainly reduced sulfhydryl groups have long been known to be important for enzyme function and cell division.

There are many obvious studies to be made with this method that we have not had time to complete, such as: What is the actual E_h and oxygen tension at the optimum level for each type of cell? What would be the effects of using perforated slides or plastic slides, or reversing the slides top and bottom, or end for end? What would be the effect of many narrow replicate slides in the same culture with spaces between, or of perforated cellophane [7] supported at a similar angle on a glass rod frame? How many cells crawl over the edges and might be caught by an underlying slide on either side? How often should the medium be changed, or the slide as a whole transplanted by simply lifting it into another bottle of medium? Can the method be used for quantitative studies of cell migration and phagocytosis by imbedding chemotactic agents in blood serum and coagulating them on the slide to give a suitable spacing and allow the cells to settle on the slide from a relatively shallow depth and then when they had settled out, filling the bottle with medium to a higher level permitting quantitative measurement of migration rate on a large scale? Will this method prove an ideal one for initial isolation of cells to be cultured? Is such a gradient in part responsible for the success of the current plasma clot, inclined roller tube, and perforated cellophane methods of culture and for the success of the anterior eye chamber [11] and intracerebral transplantation [10] techniques? Will it be possible to duplicate the ideal conditions determined by this method in a continuous flow device with perhaps automatic controls to regulate all of these factors? Will one or more layers of progressively narrower perforated cellophane strips stretched around the slide ends give a series of gradients?

While we might have waited until we had answers to many of these questions, the method seems to offer sufficient promise and there seems so much to be done with it that it appears worth while to present it in this preliminary stage so that others may work with it too.

SUMMARY

Preliminary results with a simple method of tissue culture which provides a continuous gradient in cell number, oxygen tension, oxidation-reduction potential, and cell conditioning factors are described and pictured. Cells may readily be examined *in situ* at any desired interval with any stain, or by phase microscopy in the living state. The method has proved far superior to any method hitherto tried for cultures of hemic cells of the blood and blood-forming organs. Many possible applications and variations are suggested on this simple procedure of inserting a sterile standard slide at a 45° angle into a deep suspension culture in a 1-pint wide mouth French square bottle and allowing the cells to settle out on the surface.

REFERENCES

1. Brecher, G. and Schneiderman, M., *Am. J. Clin. Pathol.* **20**, 1079 (1950).
2. Burrows, M. T., *Am. J. Physiol.* **43**, 13 (1917).
3. Carrel, A. and Lindbergh, C. A., The Culture of Organs. P. B. Hoeber, Inc., New York, 1938.
4. Earle, W. R., *Arch. exptl. Zellforsch.* **16**, 116 (1934).
5. —— *in* Visscher, M. B. (Ed.) Methods in Medical Research, Vol. 4, The Year Book Publishers, Chicago, pp. 217–218, 1951.
6. Earle, W. R., Sanford, K. K., Evans, V. J., Waltz, H. K., and Shannon, J. E., Jr., *J. Natl. Cancer Inst.* **12**, 133 (1951).
7. Earle, W. R., Sanford, K. K., and Schilling, E. E., *ibid.* **10**, 943 (1950).
8. Fieschi, A. and Astaldi, G., *Haematologica* **8**, 1 (1946).
9. Fischer, A., Fogh, B., and Bohus Jensen, A., *Acta Physiol. Scand.* **10**, 78 (1945).
10. Greene, H. S. N., *Cancer Research* **11**, 529 (1951).
11. Greene, H. S. N. and Lund, P. K., *ibid.* **4**, 452 (1944).
12. Hall, J. W. and Furth, J., *Arch. Pathol.* **25**, 46 (1938).
13. Havard, R. E. and Kendal, L. P., *Biochem. J.* **28**, 1121 (1934).
14. Lambertsen, C. J., Kough, R. H., Cooper, D. Y., Emmel, G. L., Loeschcke, H. H., and Schmidt, C. F., *J. Appl. Physiol.* **5**, 471 (1953).
15. Lewis, M. L. and Lewis, W. H., *Carnegie Inst. Wash. Contrib. Embryol.*, No. 96, **18**, 95 (1926).
16. Li, J. G. and Osgood, E. E., *Blood* **4**, 670 (1949).
17. Osgood, E. E., *Am. J. Med. Sci.* **195**, 141 (1938).
18. —— *in* A symposium on the Blood, University of Wisconsin Press, Madison, pp. 219–241, 1939.
19. Osgood, E. E. and Brownlee, I. E., *J. Am. Med. Assoc.* **108**, 1793 (1937).
20. Osgood, E. E. and Muscovitz, A. N., *ibid.* **106**, 1888 (1936).
21. Plum, C. M., *Acta Physiol. Scand.* **11**, 260 (1946).
22. Rosin, A. and Rachmilewitz, M., *Blood* **3**, 165 (1948).
23. Weil, G. C., *J. Pathol. Bacteriol.* **18**, 1 (1914).
24. Wright, G. P., *ibid.* **31**, 735 (1928).

QUANTITATIVE CYTOLOGY AND CYTOPATHOLOGY

IV. INTERFEROMETRIC MEASUREMENT OF THE ANHYDROUS ORGANIC MASS (DRY WEIGHT) OF GENETIC MATERIAL IN SPERM NUCLEI OF THE MOUSE, THE RAT AND THE GUINEA PIG

R. C. MELLORS and J. HLINKA

Division of Pathology, Sloan-Kettering Institute and Pathology Laboratories, Memorial Center for Cancer and Allied Diseases and Cornell University College of Medicine, Sloan-Kettering Division, New York, N.Y., U.S.A.

Received November 4, 1954

It is now well recognized that while the *content* of desoxyribose nucleic acid (DNA) per nucleus (or, more exactly, per chromosome set) in mammalian cells is relatively constant [9], the DNA *concentration*, when expressed as per cent of dry weight of cell nuclei, varies widely, depending to a large extent upon the method used in isolating the nuclei [2]. It is, therefore, of interest to determine the dry weight of nuclear material by a physical method, namely, microinterferometry, which avoids losses in organic mass that may accompany the use of chemical reagents as employed in the conventional procedures of cell-fractionation. Moreover, if the measurement of the dry weight is made on the nuclear material of the sperm head, the estimate has added significance; for this is a measurement of the mass, or very nearly the mass, of the genetic material which is present in a single (haploid) set of chromosomes [6]. This mass will represent the sum of two principal components, namely, protein and DNA, two lesser components, namely, ribose nucleic acid (RNA) and lipid (or lipoprotein), and, conceivably, small quantities of other substances not yet chemically characterized. After fixation and lipid-extraction of spermatozoa with alcohol and ether, the major organic materials in sperm nuclei, in so far as is known at the present time, are nucleoproteins.

The microinterferometric determination of dry weight or of anhydrous organic mass [1, 3, 4] has been made previously on unfixed sperm nuclei and on sets of chromosomes in germinal cells of the mouse testis [6]. In the present investigation this optical method is used for the analysis of fixed nuclei of spermatozoa in three mammals of the rodent order. The anhydrous organic masses, expressed in 10^{-12} g, and as ranges that statistically include 95 per cent of the estimates of the averge are 12–14, 8.5–10, and 11–12, respectively, for nuclei of fixed, lipid-extracted spermatozoa of the mouse,

the rat and the guinea pig. Since the average content of DNA per sperm nucleus (or haploid set of chromosomes) is known to be very similar and to be about 3×10^{-12} g in these species of animals, the (DNA : organic mass) ratios, assuming equivalent (about 10 per cent) random variations in the range of numerator and denominator and expressing the results both in decimal fractions and in confidence intervals, are 0.21–0.26, 0.29–0.36, and 0.24–0.28, respectively, in the nuclei of fixed spermatozoa of the mouse, the rat, and the guinea pig. On the basis of this attribute, there is evidence for a distinctive organic composition in the genetic material of the male germinal cell of the mouse in comparison with that of the rat, while a measure of the attribute in the guinea pig falls between and statistically overlaps those for the mouse and the guinea pig.

METHODS AND MATERIALS

Physical methods.—The microinterferometric procedure and the method of computation have been fully described elsewhere for the analysis of unfixed cytological material [6]. The anhydrous organic mass, M, expressed in g, is given by

$$M = \frac{\Delta}{100\,\alpha} A = \frac{\Delta}{0.19} A \tag{1}$$

where Δ, the optical path difference measured by interferometry, is in cm, A, the area of the interference pattern measured by planimetry, is in square cm; and $100\,\alpha$ is 0.18–0.19 for the principal organic (protein and nucleoprotein) constituents of cells.

In the present study cytological material which is fixed, defatted and dehydrated is also analyzed by microinterferometry. Under these conditions the formula for the computation of anhydrous mass is derived as follows.

If n_0 is the refractive index and v_0 the volume of a cell-object, then it can be shown from the equation of Gladstone and Dale [8] that

$$n_0 v_0 = n_a v_a + n_m v_m \tag{2}$$

where n_a is the refractive index and v_a the volume of the anhydrous organic material of the cell-object; n_m is the refractive index and v_m the volume of the immersion medium which penetrates the cell-object; and v_0 is set equal to the sum of v_a and v_m, that is, there is assumed to be no change of volume on mixing the two components.

From (2)

$$n_0 = n_a \frac{v_a}{v_0} + n_m \frac{v_m}{v_0} \cdot \tag{3}$$

The specific refractive increment, α, in (1) is given by

$$\alpha = \frac{n_0 - n_m}{c} \tag{4}$$

where c is the anhydrous organic mass per 100 cc volume of the cell-object.

Combining (3) and (4) yields

$$\alpha = \frac{n_a \frac{v_a}{v_0} + n_m \frac{v_m}{v_0} - n_m}{c}$$

or

$$\alpha = \frac{n_a \frac{v_a}{v_0} + n_m \frac{(v_m - 1)}{v_0}}{c} . \tag{5}$$

Now, it is assumed above that

$$v_0 = v_a + v_m$$

or

$$1 = \frac{v_a}{v_0} + \frac{v_m}{v_0}$$

and

$$\frac{v_m}{v_0} - 1 = -\frac{v_a}{v_0} . \tag{6}$$

Substituting (6) in (5) yields

$$\alpha = \frac{v_a}{v_0} \frac{n_a - n_m}{c} . \tag{7}$$

The product of the volume, v_0, and the concentration per ml, $\frac{c}{100}$, of the anhydrous organic material is equal to the anhydrous organic mass, M, of the cell-object so that

$$\frac{v_a}{v_0 \frac{c}{100}} = \frac{v_a}{M} = \bar{v}_a \tag{8}$$

where $\bar{v}_a$ is the partial specific volume or the reciprocal of the density (mass per unit volume) of the anhydrous organic material.

Combining (7) and (8) gives

$$100\,\alpha = \bar{v}_a (n_a - n_m)$$

and substituting this in (1) yields

$$M = \frac{\Delta}{(n_a - n_m)\bar{v}_a} A. \tag{9}$$

If the anhydrous organic material of the cell-object is in composition and refractivity equivalent to protein, then the average value for n_a is approximately

1.59 at wave length, 546 mμ, and that for $\bar{v}_a$ is 0.75; and when the cell-object, after dehydration, is immersed in anhydrous glycerin as a mounting medium, n_m has the value 1.47 at 546 mμ. Substituting these values in (9) gives

$$M = \frac{\Delta}{0.12 \times 0.75} A = \frac{\Delta}{0.09} A \tag{10}$$

where M is the anhydrous organic mass of the cell-object in g, Δ is the optical path difference in cm, and A is the area of the interference pattern in square cm. It is seen that the denominator in (10) has about one-half the magnitude of that in (1). Accordingly, a particular cell-object when fixed, dehydrated and mounted in glycerin will produce an optical path difference which is about one-half that observed when the cell-object is unfixed and mounted in an aqueous medium.

The optical path difference can be conveniently represented by the expression

$$\Delta = \Phi \frac{\lambda}{4} \tag{11}$$

where Φ is the phase difference expressed as a fraction or multiple of one-quarter wave length of light, $\frac{\lambda}{4}$. In the present work the monochromatic green line of the mercury emission spectrum is used for interferometry, and $\frac{\lambda}{4}$ is 0.136×10^{-4} cm.

Biological materials.—Young, sexually mature males from the Swiss-Webster albino strain of the mouse, the Wistar strain of the rat and a hybrid strain of the guinea pig were anesthesized and killed, and seminal fluid was obtained from the transected *cauda epididymis* testis. The seminal fluid was diluted by addition to several parts of Locke-Ringer solution (or of a hypotonic medium composed of equal parts of Locke-Ringer solution and distilled water), and small drops of the resulting suspension of sperm were spread on *Inconel* or aluminized[1] interference slides. For fixation, preparations were placed immediately in equal parts of 95 per cent alcohol and ether for a period of at least one-half hour and, after drying, were mounted in anhydrous glycerin (and on one occasion in hypotonic Locke-Ringer medium). Other preparations without fixation were mounted in hypotonic Locke-Ringer medium. The motility of unfixed sperm slowly diminished and finally ceased so as to permit the undertaking and the completion of the interferometric analysis within a few hours after the preparation was made. Observations on the fixed material were in some instances begun almost immediately after preparation and in almost all instances were completed on the day of preparation.

Ancillary morphological studies were made of material stained with hematoxylin and eosin and with the periodic acid-Schiff reagent in order to delineate the various structural components of the sperm. Scharlach-R was used as a fat-stain. All of the

[1] We have obtained aluminized and quartz-surfaced slides and cover slips prepared by Chemoptics, Inc., New York which have about ten per cent transmission and provide an intensity ratio of about thirtyfold between the bright and the dark fringes in the interference microscope.

TABLE I

Phase change, Φ, area, A, and anhydrous, organic mass, M, of nuclei of spermatozoa of the mouse, the rat and the guinea pig.

Spermatozoa	N	$\Phi\left(\frac{\lambda}{4}\right)$					A (μ^2)					M ($\mu\mu$g or 10^{-12} g)				
		AV	SD	$\frac{SD}{AV}$	$\frac{2SD}{\sqrt{N}}$	CI	AV	SD	$\frac{SD}{AV}$	$\frac{2SD}{\sqrt{N}}$	CI	AV	SD	$\frac{SD}{AV}$	$\frac{2SD}{\sqrt{N}}$	CI
Mouse																
Fixed	27	0.40	0.06	0.15	0.03	0.37–0.43	22	2.5	0.11	1.0	21–23	13	1.9	0.15	0.7	12 –14
Fixed*	48	0.83	0.13	0.15	0.04	0.79 0.87	22	2.7	0.12	0.8	21–23	13	1.5	0.12	0.4	13
Unfixed†	44	0.97	0.27	0.28	0.08	0.89–1.05	27	7.5	0.27	2.2	25–29	16	2.4	0.16	0.7	15 –17
Rat																
Fixed	30	0.32	0.08	0.25	0.03	0.29–0.35	20	1.3	0.07	0.5	20–21	9.3	2.3	0.25	0.8	8.5–10
Unfixed	33	0.79	0.12	0.15	0.04	0.75–0.83	19	2.9	0.15	1.0	18–20	11	2.7	0.25	1.0	10 –12
Guinea Pig																
Fixed	26	0.15	0.02	0.12	0.01	0.14–0.16	51	3.6	0.07	1.4	50–52	11.5	1.3	0.12	0.5	11 –12

Symbols and explanation.—N, number of determinations; AV, average; SD, standard deviation; CI, $AV \pm \frac{2SD}{\sqrt{N}}$, confidence interval that includes 95 per cent of the averages. Unfixed material mounted in aqueous medium; fixed material mounted in glycerin except where otherwise indicated.

* Fixed, mounted in aqueous medium.

† Data published elsewhere (6).

interferometric data reported herein pertain to the nucleus of the mature spermatozoon, as differentiated from the acrosome and other structures of the sperm head. The area of the mature sperm nucleus, expressed as per cent of that of the sperm head, is approximately 100, 90, and 50, respectively, in the mouse, the rat and the guinea pig.

RESULTS

The average magnitudes and the statistical coefficients of variation of the phase change, Φ, expressed as a fraction of one-quarter wave length of the mercury green line, the area, A, in μ^2, and the anhydrous organic mass, M, in 10^{-12} g for nuclei of spermatozoa of the mouse, the rat and the guinea pig are given in Table I.

Mouse.—The analytical data for fixed, lipid-extracted nuclei of spermatozoa are as follows (Table I): Φ, 0.40 in glycerin and 0.83 in aqueous medium; A, 22; and M, 13 with a 95 per cent confidence interval, 12–14. For unfixed nuclei M is 16 so that 19 per cent ($^3/_{16} \times 100$) of the organic mass of unfixed nuclei is fat-soluble.

Rat.—Fixed, lipid-extracted nuclei of spermatozoa have the following attributes: Φ, 0.32 in glycerin medium; A, 20; M, 9.3 with a confidence interval, 8.5–10. For unfixed nuclei, M is 11, so that 15 per cent ($1.7/11 \times 100$) of the organic mass of unfixed nuclei is fat-soluble.

Guinea pig.—The analytical data for fixed, lipid-extracted nuclei of spermatozoa are as follows: Φ, 0.15 in aqueous medium; A, 51; and M, 11.5 with a confidence interval, 11–12.

DISCUSSION

The 95 per cent confidence interval for the average anhydrous organic mass of nuclei of fixed spermatozoa of the mouse does not overlap that for the rat. Thus, on the basis of this attribute there is evidence for a quantitative difference in the organic composition of the genetic material in the male germinal cell of these two species of animals. Since the average content of DNA per sperm nucleus (or haploid set of chromosomes) is known to be very similar and to be about 3×10^{-12} g in these species of animals [9], the DNA : organic mass ratios—assuming equivalent (about 10 per cent) random variations in the range of numerator and denominator and expressing the results as confidence intervals in decimal fractions—are 0.21–0.26 and 0.29–0.36, respectively, in the nuclei of fixed spermatozoa of the mouse and the rat. If the organic materials under these analytical conditions are principally, if not entirely, nucleoproteins, then the nucleic acid : protein

ratios in the genetic material of the male germinal cell in the mouse and the rat are approximately 0.27–0.35 and 0.41–0.56, respectively. When these various attributes are considered for the guinea pig, the following data—statistically overlapping those for the mouse and questionably resolved in relation to those for the rat—are obtained: DNA : organic mass ratio, 0.24–0.28, and DNA : protein ratio, 0.32–0.39.

Further work along these lines, together with analyses of the organic mass of sets of chromosomes such as are now being performed [5, 6], should provide information concerning the homology or heterology of chromosomes [7] in various species of animals and in various types of cells, e.g. in germinal, somatic or neoplastic cells in the same animal.

SUMMARY AND CONCLUSIONS

1. Further biological applications of interference microscopy are undertaken. Equations are derived for the computation of the anhydrous organic mass, M, in fixed, lipid-extracted cytological material when mounted in a medium, such as glycerin, of known refractive index.

2. The average values for M, expressed in 10^{-12} g, are 13.0, 9.3 and 11.5, respectively, for nuclei of fixed, lipid-extracted spermatozoa of the mouse, the rat and the guinea pig.

3. The significance of these data are analyzed statistically, and the data (along with known information concerning the desoxyribose nucleic acid content of sperm nuclei) are discussed in terms of the quantitative and qualitative organic composition of genetic material in these species of mammals.

This investigation was supported in part by research grant C-1345 from the National Cancer Institute of the National Institutes of Health, Public Health Service and was undertaken during the tenure of a Damon Runyon Memorial Fund grant.

REFERENCES

1. Barer, R., *Nature* **169**, 366 (1952).
2. Davidson, J. N., The Biochemistry of the Nucleic Acids, p. 89. 1st. ed., Methuen and Co., Ltd., London, 1950.
3. Davies, H. G. and Wilkins, M. H. F., *Nature* **169**, 541 (1952).
4. Mellors, R. C. Kupfer, A., and Hollender, A., *Cancer* **6**, 372 (1953).
5. Mellors, R. C. and Stoholski, A. (In preparation.)
6. Mellors, R. C., Stoholski, A., and Beyer, H., *Cancer* **7**, 873 (1954).
7. Mirsky, A. E. and Ris, H., *Nature* **163**, 666 (1949).
8. Partington, J. R., An Advanced Treatise on Physical Chemistry. Vol. 4, p. 72. Longmans, Green and Co., London, 1953.
9. Vendrely, R. and Vendrely, C., *Experientia* **5**, 327 (1949).

Experimental Cell Research, **9**, *135–138 (1955)*

OSCILLATIONS OF OOCYTE NUCLEI IN THE OVARIOLES OF THE GRASSHOPPER *MELANOPLUS DIFFERENTIALIS DIFFERENTIALIS (ORTHOPTERA, ACRIDIDAE)*

T. N. TAHMISIAN, A. M. BRUES, G. SVIHLA and ELEANOR H. SLIFER

Division of Biological and Medical Research, Argonne National Laboratory, Lemont, Ill., and Department of Zoology, State University of Iowa, Iowa City, Iowa, U.S.A.

Received November 4, 1954

The ovarioles of the grasshopper *Melanoplus differentialis differentialis* (Thomas) contain growing primary oocytes with nuclei that may attain a maximum diameter of 150 μ. It has been observed that these nuclei oscillate slowly during the period of yolk deposition which may take 45 days or longer. So far as the authors are aware this is a type of motion which has not been recorded previously for the cells of any other biological material. This paper describes attempts to define further the nature and conditions of this motion by means of direct observation and by time-lapse cinematography. It was originally reported in two abstracts [4, 5].

MATERIALS AND METHODS

Ovaries of adult and nymphal grasshoppers were used in these studies. Whole ovaries of first and second instar nymphs were dissected in Ringer's solution buffered with M/15 phosphate at pH 6.8 and compressed gently on a "Roto-compressor" slide[1] until a single layer of ovarioles was observed under the dissecting microscope. In handling older nymphs and adults the ovaries were placed in Ringer's solution and the ovarioles separated. One ovariole was cut free at the oviduct and at the median ligament and the outer layers which envelop the ovariole were then peeled back to improve conditions of observation. This ovariole was then compressed on a "Roto-compressor" slide until the largest egg came into contact with the slide and cover glass.

The earliest eggs were photographed with a 2 mm 1.32 N.A. objective using a Leitz phase contrast system set for dark phase and a 10× ocular. In photographing the larger eggs it was necessary to employ objectives of lower power. The light source was a 6 volt General Electric bulb 30 G 12/15 operated at 5 amperes. The frosted lens of the lamp housing (Ortholux) was polished to allow Koehler illumination. This was found to increase light transmission about 30 per cent. The micro-

[1] The "Roto-compressor" slide was purchased from the Biological Institute, 2007 Broad Street, Philadelphia, Pennsylvania.

scope was partially surrounded by a box made of $^1/_2$-inch lucite which was controlled thermostatically so that the object, as well as the objectives, stage and condenser, were maintained at 0.1°C of the desired temperature. Except where otherwise noted the observations were made at 28°C. Cinematographs were taken on 16 mm Super X Eastman film. The electronic circuit for lapsed-time records was so designed that the cells were illuminated for a period only slightly longer than was necessary for photographic exposure.

RESULTS

In the early primary oocytes no nuclear oscillations were observed. The individual chromosomes, however, are in active motion which is rapid enough to be discerned by direct observation. Using lapsed-time photography it can be seen that they bend and move independently of each other.

Beginning about the time of the third nymphal instar motions of the whole nucleus are seen. At first the movement is irregular but as the oocyte enlarges it becomes rhythmic and regular. The time required for one complete oscillatory cycle varies between five and forty-five seconds as determined by direct observation and motion picture records show that the rate of oscillation of a given nucleus continues approximately the same for hours. Measurements of the angle traversed during each oscillation were made from films and were found to vary within the limits of five degrees and twenty degrees. The amplitude of oscillation, like the period, tends to remain the same for hours in a given egg.

The oscillations always occur about a short axis of the egg. In other words, the axis of nuclear rotation is perpendicular to the longest axis of the egg and may lie in a vertical plane, in a horizontal plane, or in a plane intermediate between these. Oscillation about a vertical axis was observed most frequently and attempts were made to alter the plane of oscillation by changing the orientation of the egg to natural gravitational force. Changing the orientation by 45° and 90° for as long as two and a half hours was ineffective.

The oscillation itself is sensitive to temperature. In a few experiments where the temperature of the system was lowered to 8°C the period was greatly reduced but the oscillations continued. At a temperature of 38°C the rate of oscillation was increased but temperatures higher than this were lethal. Pressure on the egg increased the oscillation rate and at the same time decreased the amplitude.

A number of nuclei were dissected free from the egg and observed (i) in Ringer's solution; (ii) in isotonic saline devoid of sodium and buffered

at pH 6.8 to 7.8 with potassium phosphates; and (iii) in isotonic sucrose and proteose-peptone devoid of salts. In no case did an isolated nucleus show movements of any kind.

The nature of the oscillatory motion can be likened to that of the balance wheel of a watch; that is, the maximum speed appears to be attained in the middle of the excursion with abrupt deceleration near the end. No microscopic evidence was seen of any structural element limiting the excursion, in spite of the uniformity of the movement from one oscillation cycle to the next.

DISCUSSION

So far as is known no previous investigator has described the rhythmic oscillation of nuclei. Pomerat [2, 3] has reported revolving nuclei in tissue cultures of human nasal mucosa which were studied with the aid of cinematography. These appear to undergo occasional large excursions which are not rhythmical in nature and which are too slow to be observed directly. One of us (E. H. Slifer) was present at the Leiden meeting of the VIIIth International Congress for Cell Biology and heard Krantz [1] present a paper on nuclear rotation and pulsation in cultures of epithelial cells from the kidney of a new-born mouse. Here, too, time-lapse cinematography was necessary in order to detect the nuclear movements.

We have no evidence as to the source of the energy expended in the oscillation process or of the way in which it is translated into motion. We have also been unable to throw light on the way in which this motion is subject to such specific restrictions. We have found that the degree of motion is sensitive to temperature and is affected by the application of pressure.

It may be recalled that the grasshopper egg, during its development, produces a large amount of yolk. In accordance with current views regarding the production of cytoplasmic materials it is conceivable that the nuclear motion is the result of the extrusion of material from the nucleus into the cytoplasm. Functionally, it is obvious that the oscillations may be important in promoting diffusion within the nucleus and between nucleus and cytoplasm. Finally, since rotational or oscillatory movements of the nucleus are now known to occur in the cells of both vertebrates and invertebrates, it seems probable that nuclear motion has some general physiological significance. Further questions concerning the mechanism and functional importance of these movements are under investigation.

SUMMARY

1. Oscillatory movements of the nucleus of the grasshopper oocyte have been observed directly and recorded cinematographically.

2. In young oocytes from first and second instar grasshopper nymphs, independent movements of the chromosomes occur. Arrhythmic movements of the nucleus are first observed in oocytes from third instar females. In older oocytes these movements become rhythmic and oscillatory with a period of from 5 to 45 seconds at 28°C and with an excursion of from 5 to 20 degrees. The motion resembles that of the balance wheel of a watch. The axis of rotation lies perpendicular to the long axis of the egg.

3. The nuclear oscillations are affected by temperature and pressure but the plane of oscillation is not influenced by natural gravitational forces.

REFERENCES

1. Krantz, H., Program VIIIth International Congress for Cell Biology, Leiden, The Netherlands, Sep. 1–8, 1954.
2. Pomerat, C. M., *Exptl. Cell Research* **5**, 19 (1953).
3. —— *Excerpta Medica* **8**, 422, abs. 1751 (1954).
4. Tahmisian, T. N., Brues, A. M., and Svihla, G., *Am. J. Physiol.* **171**, 772 (1952).
5. Tahmisian, T. H. and Svihla, G., Progress Report, Argonne National Laboratory Quarterly Report, Nov.–Dec., 1951 and Jan., 1952, ANL-4745.

THE LOCALISATION OF A PERMEABILITY BARRIER IN THE CELLS OF *BACILLUS MEGATERIUM*

C. WEIBULL

Institute of Biochemistry, University of Uppsala, Uppsala, Sweden

Received November 5, 1954

The existence of permeability barriers in bacterial cells has been ascertained several times. The localisation of these barriers is less well known. This paper describes the permeability of the cell wall and the protoplasmic surface of living *Bacillus megaterium* cells to various solutes surrounding the cells in a bacterial suspension.

MATERIALS AND METHODS

B. megaterium.—Strain KM (6) was used in all experiments. Cultures for experimental work were grown in 2 per cent Difco peptone at 30°C with mechanical agitation using Erlenmeyer or Fernbach flasks. The cells were harvested during the phase of declining growth rate (6–12-hour cultures).

Optical and photographical equipment.—An Ortholux microscope with an apochromatic 90 × oil immersion objective, N.A. 1.10, was used. Photographs were taken at an original magnification of 1000–1300 diameters using a 8 × eyepiece and Ilford 27° panchromatic plates. The photographs published in this paper were taken without phase contrast. For direct measurements of particles under the microscope a Leitz ocular micrometer, equipped with a 12.5 × eyepiece, was used.

Analytical methods.—Nitrogen determinations were performed according to the Kjeldahl method, phosphorus according to Allen [1].

Ultraviolet absorption measurements were made with a Beckman model DU spectrophotometer.

The dry weight of bacterial suspensions was determined by drying samples at 100° C.

The number of bacteria in a suspension was determined after suitable dilutions in a Petroff-Hauser counting chamber. Forty squares, each comprising 1.25×10^{-6} ml and about 200 cells, were counted.

The partial specific volume of bacterial cell substances in water was determined by the pycnometer method [9].

EXPERIMENTAL RESULTS

The investigation was planned as follows. First the morphological differentiation between cell wall and protoplasm in *B. megaterium* cells was

investigated under the light microscope (section *a*, below). On the basis of these observations and by measurements on a great number of living cells the average volume of one cell, v_c, and of the included protoplasm, v_p, was determined. Counts gave the number of cells, n, in 1 ml of a given suspension. The total volume occupied by the bacterial cells $= v_c \times n$, and by the protoplasm, $= v_p \times n$, was thus obtained. A determination of the bacterial dry weight/ml, w, gave the ratios cell volume/cell dry weight $= v_c \times n/w = R_1$ and volume of protoplasm/cell dry weight $= v_p \times n/w = R_2$. The results are given in section *b* below.

The permeability of resting *B. megaterium* cells to various solutes under semianaerobic conditions was determined by adding to a heavy bacterial suspension (volume V_1 ml, dry weight content W mg/ml, kept in a narrow test tube) a solution (volume V_2, solute concentration c) of the substance to be investigated. After some time the cells were precipitated by centrifugation and the concentration c_s of the solute determined in the supernatant.

We get the space not permeated by the solute in the final suspension

$$v^t_{\text{imp}} = V_1 + V_2 - V_2 c/c_s.$$

From the dry weight determination we get the ratio $R_3 = v^t_{\text{imp}}/W \times V_1$.

A somewhat more elaborate, but in principle identical, technique was applied when the permeability of cells in aerated and dilute suspensions was determined. The details will be given below.

A value for $R_3 - R_1$ and $> R_2$ indicates that the solute tested does not at all penetrate into the cell. The case $R_1 > R_3 > R_2$ is most easily explained by assuming that the solute permeates the cell wall more or less completely but not the protoplasm. $R_3 < R_1$ and R_2 would indicate a penetration of both the cell wall and the protoplasm.

The experimentally found values are given in section *d* below.

a. Morphological differentiation between cell wall and protoplasm.—The nature of the cell wall as a rigid structure, determining the shape of a bacterium has been demonstrated by Salton and Horne [9]. They were able to isolate pure cell walls by disruption of the cells and subsequent centrifugations of the resulting suspension. Murray and Robinow [5] have published pictures of *Bacillus cereus* cells showing that the protoplasm can be squeezed out of the cells leaving the cell wall as an empty structure of the same outer shape as the original cells. The present author [11] treated the cells with lysozyme in 0.2 *M* sucrose. It was concluded from analyses of the surrounding medium that the cell wall was dissolved under these con-

ditions but that the remaining spherical bodies represented the protoplasmic constituents, preserved as an entity after removal of the cell wall. These bodies were consequently named protoplasts.

It can be demonstrated directly in the light microscope that the cell wall is actually removed by the lysozyme by comparing cells and protoplasts suspended in dilute indian ink. The rod shaped body in Fig. 1 is an untreated *B. megaterium* cell photographed in this medium. The cell consists of a central part, surrounded by an outer, somewhat brighter layer or envelope. It can be seen from Fig. 2 that this envelope represents the rigid cell wall. Here cells have been squeezed between slide and cover slip. In part of the cells the protoplasmic contents have left the cell and have been replaced by the dark indian ink. The bright outer layer remains unchanged and obviously represents the cell wall. This is particularly evident from the appearance of those bacteria which are only partly empty. Both the unchanged and the empty parts are surrounded by the same bright envelope. The envelope remains unchanged after boiling and after fixing in 4 per cent formaldehyde.

When protoplasts, prepared according to [11] and fixed in 4 per cent formaldehyde, are suspended in indian ink no bright envelope around the spherical bodies can be seen, Fig. 1. This shows that the cell wall is removed by the lysozyme, leaving the protoplasm in the form of spherical bodies.

b. Cell dimensions of B. megaterium. Counts and dry weight determinations. Volume/dry weight ratios.—In this section the experimentally found values for the quantities v_c, v_p, n, R_1 and R_2 are given.

The average cell width was calculated from measurements on about 100 living unstained cells, suspended in a mixture of indian ink and 0.02 *M* phosphate buffer (*c.* 1:4). Identical values were obtained when the phosphate was substituted for 0.2 *M* sucrose or 0.6 *M* urethan.

The most rapid and accurate way of determining the average length of the bacteria was found to be the measurement of 600–800 bacteria on the photographical plates. The plates were calibrated with an objective micrometer.

The shape of the bacteria was found to be very nearly that of a cylinder with spherical ends. Some bacteria, near the dividing point, assumed the shape of two or more cylinders, linked together and each having spherical ends. The relative number of such bacteria was determined. Since no correlation between length and width of a bacterium was found, the average volume of one bacterium was easily calculated from these dimensions.

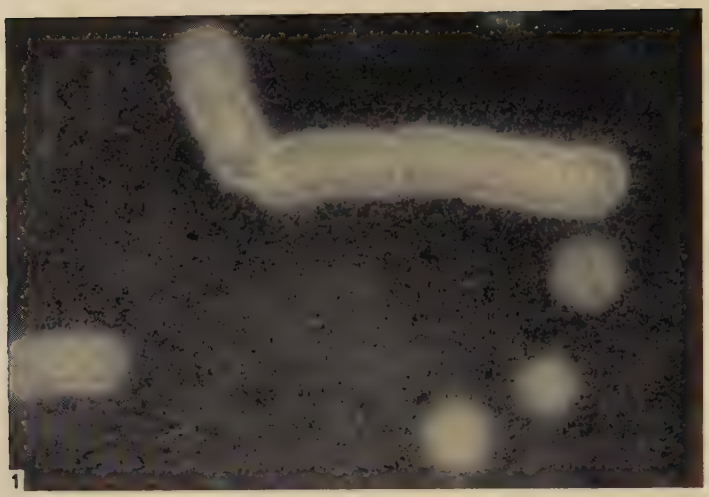


Fig. 1. Cells and protoplasts of *B. megaterium* in dilute indian ink. Magnification 3800 .

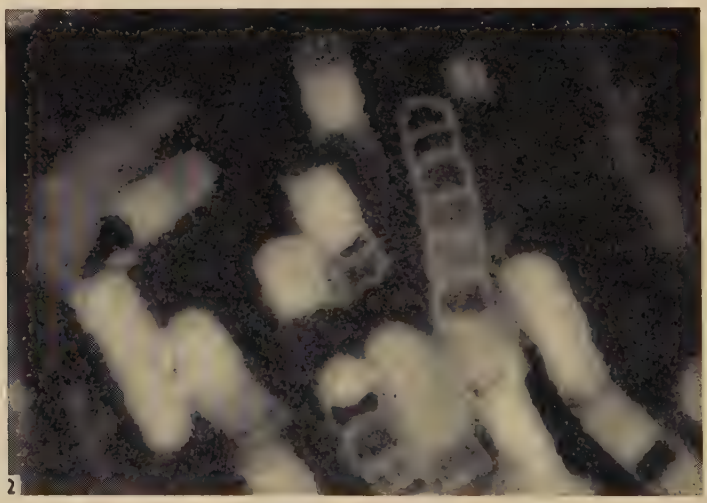


Fig. 2. Cells of *B. megaterium* sqeezed between slide and cover slip. Medium: dilute indian ink. Magnification 3800 ×.

(Note: One bacterium means one organism seen as a free entity separated in space from all others. One bacterium may consist of two or more cylinders, but these are then still linked together.)

The experimentally found values for the various quantities are given in Table I. All error limits are given as statistical standard deviations. The

error limits of the bacterial volumes have been obtained by multiplication of the corresponding maximal and minimal length, width and number values. A maximal "error volume" is thus obtained.

The ratio total dry weight/total wet weight was obtained from the ratio total volume/total dry weight and from a determination of the average partial specific volume of the material, except water, that builds up the

TABLE I

	Nature of bact. preparation		Average
	I. Early phase of decl. growth rate	II. Late phase of decl. growth rate	
Bacterial length, mm	$(8.77 \pm 0.12) \times 10^{-3}$	$(11.11 \pm 0.11) \times 10^{-3}$	
Bacterial width, total, mm	$(2.184 \pm 0.010) \times 10^{-3}$	$(2.107 \pm 0.008) \times 10^{-3}$	
Width of protoplasm, mm	$(1.648 \pm 0.009) \times 10^{-3}$	$(1.672 \pm 0.009) \times 10^{-3}$	
*	1.27	1.53	
Total vol. of one bact., $\mu l = v_c$	$(29.38 \pm 0.72) \times 10^{-9}$	$(35.04 \pm 0.66) \times 10^{-9}$	
Vol. of protoplasm, $\mu l = v_p$	$(15.64 \pm 0.50) \times 10^{-9}$	$(20.91 \pm 0.49) \times 10^{-9}$	
Number of bact./ml $= n$	$(2.000 \pm 0.042) \times 10^{9}$	$(0.966 \pm 0.018) \times 10^{9}$	
Total dry weight, mg/ml susp. $= w$ . .	11.94	8.30	
Total vol., μl, of bact./ml susp. $= v_c \times n$	58.76 ± 2.67	33.86 ± 1.26	
Total vol., μl, of protopl./ml susp. $= v_p \times n$	31.25 ∓ 1.51	20.21 ± 0.84	
Total bact. vol./total dry weight $= R_1$.	4.92 ± 0.22	4.08 ± 0.15	4.50
Vol. of protopl./total dry weight $= R_2$.	2.61 ± 0.13	2.44 ± 0.10	2.53
Total dry weight/total wet weight . .	0.192 ± 0.009	0.232 ± 0.011	0.212

* This line gives the average number of cylinders, each with spherical ends, which builds up one bacterium (see text).

B. megaterium cells. A value of 0.70 was found for the latter quantity. As is seen from Table I, an average value of 0.212 is obtained for the ratio total dry weight/total wet weight. Many determinations of this ratio have been made earlier [7]. The method used in these earlier determinations consists in the preparation and weighing of a paste of wet bacteria and in the determination of the dry weight of the paste after heating a sample at 100°C. Values of 0.15–0.30 have been obtained. For *B. megaterium* a value of 0.203 has been reported [3]. This procedure can, however, hardly give very reliable results, since it is very difficult to prepare a paste of wet bacteria that does not contain any external water. On the other hand some internal moisture may be removed when the bacteria are pressed, blotted and so on [3].

c) Permeability experiments.—Two experiments (*I* and *II*) with phosphate buffers ($NaH_2PO_4 + Na_2HPO_4$), pH 7.2 will be described in detail. The first one was performed with a heavy bacterial suspension under semianaerobic conditions, in the second one the suspension was aerated after previous dilution. The symbols refer to formula (1).

TABLE II

Permeability experiments with phosphate buffer, pH 7.2. For explanations, see text.

Phosphate concentration, *M*		
Buffer = *c*	Supernatant	Blank
0.200	0.0463	0.0006

I. To 4.00 ml (V_1) of a suspension of *B. megaterium* cells ($W = 54.6$ mg/ml), washed twice in distilled water, 1.00 ml (V_2) of 0.2 *M* phosphate buffer, pH 7.2, was added. The mixture was left standing for 2 hours. The suspension was then centrifuged together with a blank to which water instead of phosphate had been added. The supernatants were analyzed for inorganic phosphate with the original buffer as a standard. The analytical figures are given in Table II.

We get $v^t_{imp} = 4 + 1 - 0.200 \times 1/(0.0465 - 0.0006) = 0.622$ ml.

$R_3 = 0.622/0.0546 \times 4 = 2.85$.

II. 4.00 of a bacterial suspension, 5.00 ml of 0.2 *M* phosphate buffer, pH 7.2, and 90 ml of water were poured in a Fernbach flask and aerated for 1 hour at 30°C by means of mechanical agitation. The suspension was then quantitatively transferred to centrifuge tubes. After centrifugation the precipitated cells were quantitatively transferred to a graduated test tube with the help of some water and again centrifuged. All supernatants were united and evaporated to dryness. The residue was dissolved in a small amount of water and quantitatively transferred to the graduated tube containing the bacterial cells. The total volume of cells + phosphate + wash water was 7.25 ml. The tube was centrifuged and the supernatant was analysed for inorganic phosphate with the original buffer as a standard. The concentration = c_s was determined to 0.1582 *M*. A blank, to which no phosphate was added, but which was otherwise treated in an identical way, gave $c_s = 0.0024$.

We get $v^t_{imp} = 7.25 - 5 \times 0.200/(0.1582 - 0.0024) = 0.83$ ml.

The precipitated bacteria were washed and weighed after heating at 100°C. The dry weight was found to be 0.299 g.

Thus we get $R_3 = 0.83/0.299 = 2.78$.

The experiment now described was repeated but now 1 ml 1 *M* glucose was added before the seration. A value of 1.04 for R_3 was obtained in this case.

TABLE III

Permeability experiments with sucrose solutions. For explanations, see text.

Bact. dry weight, mg/ml final susp.	Dry weight, mg/ml supernatant	Dry weight, 1 *M* sucrose, mg/ml	$v^t_{imp.}$/bact. dry weight (R_3)
47.6	79.8	342	3.00
63.2	85.3	342	3.12
53.3	81.4	342	3.04

TABLE IV

Values for the ratio $R_3 = v^t$ imp./WV_1, determined for various solutes. For explanations, see text.

Solute	Time of contact, solute-cells, min.	c_s, *M* approx.	R_3
Phosphate, pH 7.2, not aerated	20	0.04	2.78
,,	120	0.04	2.85
,,	20	0.15	2.92
Phosphate, pH 7.2, aerated	60	0.10 (015)*	2.78
Phosphate, pH 7.2, aerated with gluc.	60	0.10 (015)*	1.04
Sucrose	120	0.2	3.05
DPN	60	0.002	3.02
,,	60	0.001	2.82
Urethan	20	0.6	1.49

* See text.

From experimental data given in an earlier paper [11] the permeability of the *B. megaterium* cells to sucrose can be determined. The sucrose was added to a final concentration of 0.2 *M*. A Fehling test showed that less than 1 per cent of the sucrose was converted to reducing substances during the experiment. Table III gives the results.

The permeability of the bacterial cell to urethan and diphosphopyridine nucleotide (DPN) was also determined. The concentration of DPN (Sigma

cozymase "90") was determined by means of its light absorption in the ultraviolet range. The concentration of urethan was determined by Kjeldahl analyses.

The values of the ratio R_3 for phosphate buffer, pH 7.2, sucrose, DPN and urethan are given in Table IV.

DISCUSSION

The values for the quantity R_3 = impermeable volume per g bacterial dry weight, reported above, fall into two groups. In the first group R_3 varies between 2.8−3.1, in the second one R_3 is smaller than 1.5.

Let us consider the first case ($R_3 = 2.8-3.1$, i.e. $R_1 > R_3 > R_2$).

If a permeability barrier were present at the surface of the *cell wall*, the values obtained for R_3 could only be explained by assuming that the solutes slowly leak into the cell. This explanation is highly improbable when the results of experiments with cells suspended for different times in identical solutions are considered. The corresponding R_3 values are the same within the error limits (Table IV).

The experiments could be explained without the concept of permeability barriers by assuming that only a small fraction of the space within the cell is accessible to the solutes. This explanation, too, seems not very probable in the light of the R_3 value obtained for urethan. Moreover, the presence of glucose in an aerated suspension makes R_3 for phosphate change from 2.8 to 1.0 (Table IV).

The experiments are therefore most easily explained by assuming a permeability barrier at the surface of the *protoplasm*. The average value for the impermeable volume, 2.9 ml per g bacterial dry weight, is slightly higher than the corresponding values for the volume of the protoplasm, 2.5 ml. The difference, 0.4 ml, represents probably the part of the cell wall (total volume 2.0 ml) which is not permeated by the medium, i.e. the cell wall material itself and bound water.

The barrier is impermeable to phosphate buffer, pH 7.2, when only endogenous respiration is going on, but a net transfer of phosphate takes place when glucose is metabolized. These findings are in accordance with the experiments of Mitchell and Moyle [4], performed with *Micrococcus pyogenes* cells. A certain part of the space enclosed within these cells was found impermeable to phosphate ions provided the cells were resting.

Under semianaerobic conditions (the cells are kept in narrow test tubes, bacterial dry weight/ml suspension is higher than 50 mg) the protoplasmic

surface is impermeable or only slowly permeable to phosphate buffers, sucrose and DPN but permeable to urethan.

The existence of a permeability barrier at the protoplasmic surface is in accordance with the well-known plasmolysis phenomenon, described in connection with a great number of organisms, including bacteria. It has, however, not been possible to plasmolyse *B. megaterium* cells [2].

The existence of a permeability barrier at the protoplasmic surface has been demonstrated in fixed cells of *B. cereus* [8]. It was demonstrated that Victoria blue stains penetrated freely through the cell wall but was retarded at the surface of the protoplasm. These findings are thus in accordance with the results of the experiments with living cells described in this paper.

The performed experiments do not rule out an exchange of the investigated solutes between cells and medium taking place without any net transfer. Mitchell and Moyle [4] have shown that such an exchange actually occurs between *M. pyogenes* cells and the surrounding medium. This is probably true, at least for some solutes, in the case of *B. megaterium* as well.

SUMMARY

Determinations of the ratios total volume/total dry weight and volume of protoplasm/total dry weight have been performed with cells of *Bacillus megaterium*. On the basis of these measurements the permeability of the cells to various solutes has been investigated. The experimental results are most easily explained by assuming a permeability barrier at the surface of the protoplasm. The cell wall is in all probability permeable to the solutes.

The author thanks Prof. A. Tiselius for his kind interest in this work, which was performed at his Institute.

The work is part of a program on the submicroscopic structure of the bacterial cell, which is financially supported by the Swedish Natural Science Council.

REFERENCES

1. Allen, R. J. A., *Biochem. J.* **34**, 858 (1940).
2. Fischer, A., *Z. Hyg. Infektionskrankh.* **35**, 1 (1900).
3. Henry, B. S. and Friedman, C. A., *J. Bacteriol.* **33**, 323 (1937).
4. Mitchell, P. and Moyle, J. M., *J. Gen. Microbiol.* **9**, 273 (1953).
5. Murray, R. G. E. and Robinow, C. F., *J. Bacteriol.* **63**, 298 (1952).
6. Northrop, J. H., *J. Gen. Physiol.* **34**, 715 (1951).
7. Porter, J. R., Bacterial Chemistry and Physiology. John Wiley and Sons, Inc., New York, p. 355, 1947.
8. Robinow, C. F. and Murray, R. G. E., *Exptl. Cell Research* **4**, 390 (1953).
9. Salton, M. R. J. and Horne, R. W., *Biochim. et Biophys. Acta* **7**, 177 (1951).
10. Svedberg, T. and Pedersen, K. O., The Ultracentrifuge. Clarendon Press, Oxford, p. 58, 1940.
11. Weibull, C., *J. Bacteriol.* **66**, 686 (1953).

ISOLATION AND ANALYSIS OF THE LARGE CYTOPLASMIC GRANULES OF TISSUE MAST CELLS[1]

A. HEDBOM and O. SNELLMAN

Institute of Biochemistry, University of Uppsala, Sweden

Received November 13, 1954

Holmgren and Wilander [10, 27] presented evidence that normal tissue mast cells almost certainly contain heparin in their cytoplasm. Further, it was claimed by these and other authors, compare [1], that the heparin is definitely located in the microscopically visible large mast granules, which was supposed to explain the "metachromasia of large granules" demonstrated by Ehrlich as early as in 1877 [6]. This opinion has been widely accepted [1, 12]. However, later data based on a more careful cytological reinvestigation of the microsome-like intergranular material have cast some doubt on the validity of this localization of heparin [13]. Instead, the results indicate that the heparin-bearing native complex (a heparin lipo-protein) probably is located *not* in the large granules, but within the intergranular cytoplasm.

In this paper further fractionation experiments and chemical data on the composition of isolated and washed large mast cell granules are presented.

MATERIAL AND METHODS

Fresh frozen ox liver capsules were thawed and carefully cleaned from adherent liver tissue, then frozen in solid CO_2 and ground in a handmill fitted with grooved metal plates, designed for the grinding of spices. The frozen fine granulated material obtained in this way was very lightly ground in a porcelain mortar for 5 minutes in order to break up the cell membranes. Occasionally one-ml portions of 0.88*M* sucrose were added. This process yielded a mass of red gelatinous tissue, which was suspended and diluted to four times the original volume by the addition of 0.25*M* sucrose.

During the whole process the pH was maintained between 7.0–7.2 by adding small amounts of 0.05*M* NaOH. If this was neglected, the mixture tended to become more and more acid, and in such cases heparin precipitated.

Centrifugal fractionation.—The mast cell homogenate was fractionated according to the procedure shown in Fig. 1. All centrifugations were performed in an International refrigerated centrifuge at a temperature of + 2°.

[1] The following abbreviations are employed in this communication:– DNA: deoxyribonucleic acid; RNA: ribonucleic acid.

First the nuclei were sedimented at 800 × gravity (*g*) for 6 minutes. The sediment P_1 containing intact mast cells, free nuclei and a few red blood cells was discarded. The extract, which contained the elements derived from the mast cell cytoplasm, was then centrifuged two cycles of 25 minutes at 800 × *g*. The supernatant S containing submicroscopic particulate material was retained for other investigations. The opaque sediment P_2 was resuspended in 0.25*M* sucrose.

In order to remove the last traces of nuclei, a method described by Hogeboom *et al.* [9] was found suitable. The extract was carefully layered over a small volume of 0.88*M* sucrose. After centrifugation at 800 × *g* for 6 minutes, the last unbroken cells and nuclei could be found in the sediment P_3 and removed. The extract was then centrifuged for 25 minutes at 800 × *g*. The sediment P_4 consisted of three sharply defined components. The light yellow lipoid layer at the top was readily removed and discarded. The light brown fluffy layer in the middle contained a concentrate of large granules with only few impurities. The bottom layer contained some granules but mainly a dense grey protein gel which was discarded.

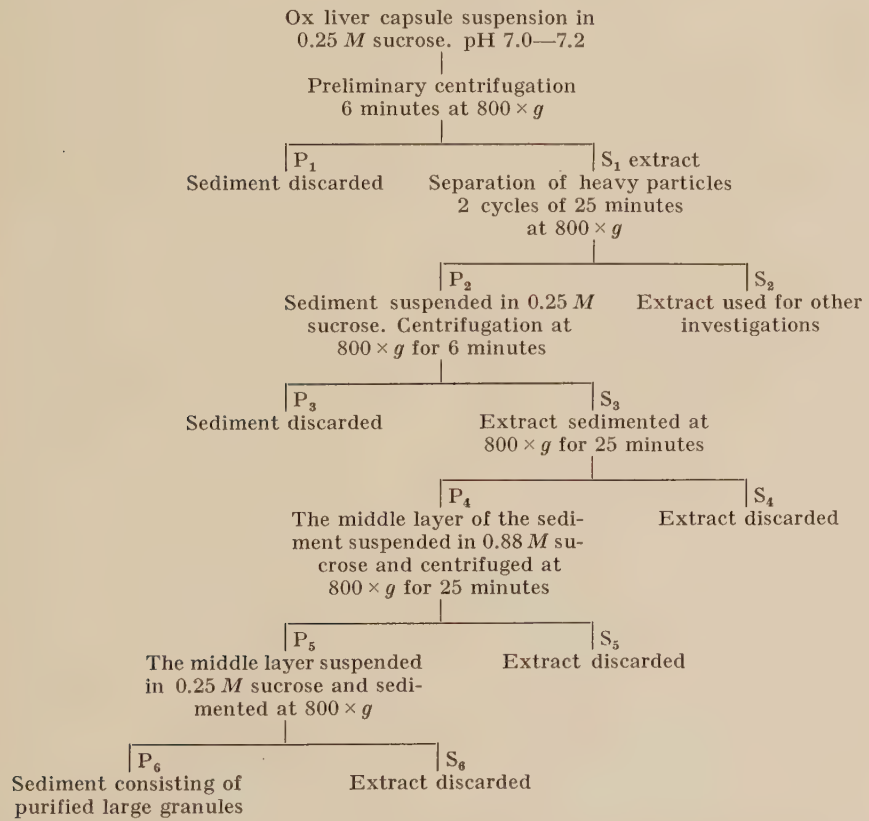

Ox liver capsule suspension in 0.25 M sucrose. pH 7.0—7.2
Preliminary centrifugation 6 minutes at 800 × g
P1
Sediment discarded
S1 extract
Separation of heavy particles 2 cycles of 25 minutes at 800 × g
P2
Sediment suspended in 0.25 M sucrose. Centrifugation at 800 × g for 6 minutes
S2
Extract used for other investigations
P3
Sediment discarded
S3
Extract sedimented at 800 × g for 25 minutes
P4
The middle layer of the sediment suspended in 0.88 M sucrose and centrifuged at 800 × g for 25 minutes
S4
Extract discarded
P5
The middle layer suspended in 0.25 M sucrose and sedimented at 800 × g
S5
Extract discarded
P6
Sediment consisting of purified large granules
S6
Extract discarded

Fig. 1. Diagram of flow for the isolation of cytoplasmic large granules from tissue mast cells.

The middle layer was then suspended in 0.88*M* sucrose and again sedimented at 800 × *g*. Only a very small amount of the two other components could be detected. After a final suspension in 0.25*M* sucrose and sedimentation at 800 × *g*, it was possible to obtain a uniform concentration of purified large mast cell granules without adsorbed protein gel and with only negligible amounts of microsomes.

From 100 g of liver capsules (wet weight), 5–7 g of P_2 and 300–500 mg of large granules were obtained.

In some preparations, when the phosphorus content was not of special interest, the original material after grinding was suspended and fractionated in isotonic phosphate buffer. Contrary to the observations of Hogeboom, Schneider and Palade [9], the large granules did not seem to aggregate in this case.

A preparation with a veronal buffer gave similar results.

Attempts to secure a better yield by using zone electrophoresis in starch or paper pulp or by adsorption on Celite were unsuccessful.

The appearance and character of washed large granules (*L.G.*).—The final product thus obtained was found by microscopy to consist of round particles with a diameter of 0.2–0.5 μ. These seemed to be different from liver mitochondria which are rodlike [9]. Mast cell granules show a high power of refractivity and should for that reason be visible in an ordinary microscope. They are, however, small in size and therefore hardly visible without staining. They maintain their size and shape for some time in the pH interval 6–8, but aggregate between pH 3–6, and outside of these intervals, they are disintegrated.

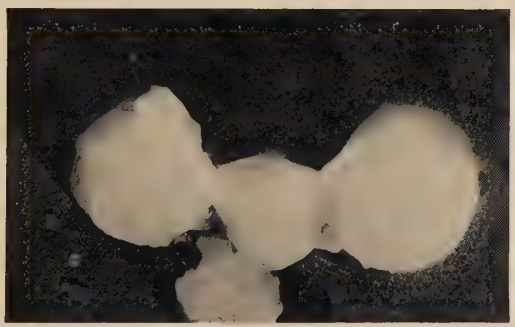
Supravitally stained with Azur A they are distinctly visible as orthochromatic-blue spherical particles. In samples inspected at early stages of the preparation, the blue granules were imbedded in a metachromatic-red protein gel. The washed L.G. were found to be practically free from metachromatic material. After staining with Janus Green B in a concentration of 1:500,000, the large granules appeared as blue-green particles. This is consistent with the observed staining properties of mitochondria of other materials [22].

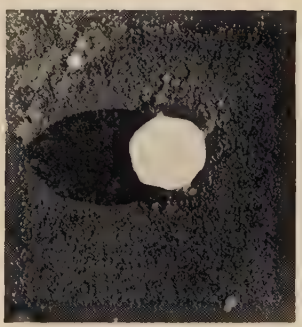
Electron-microscopic investigation.—Small dust-like droplets from a diluted suspension of purified L.G. were sprayed on an electron microscope holder precooled with solid carbon-dioxide. The material was thus immediately frozen and subsequently dried in a freezing drying apparatus. After gold-manganine shadowing. the sample was examined in a Siemens electron microscope.

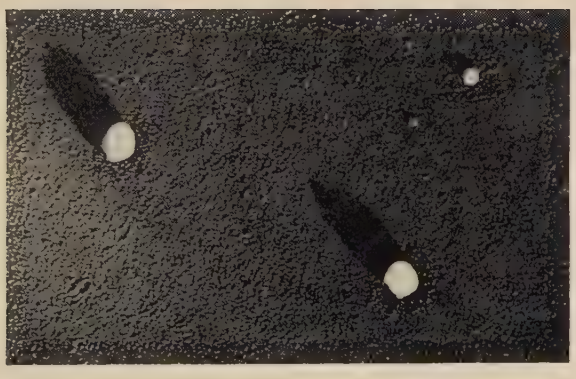
In the preparation, well-preserved large granules were present in the form of spherical particles with a diameter between 0.3 and 0.5 μ (Fig. 2). Occasionally these were grouped in close proximity, but more frequently the distribution was distinct and widely separate. The internal structure of the granular body was not visible, and the existence of an enveloping membrane could not be discerned.

Chemical investigation of the L.G.-fraction.—A sample of the washed fraction was separated into three major groups of compounds according to generally accepted methods for tissue analysis.

Fig. 2. Cytoplasmic large granules from tissue mast cells isolated in 0.25*M* sucrose solution. Shadow casting with gold manganine. Original magnification × 8500. Final magnification × 43,000.







I. The sample was treated with cold trichloroacetic acid (TCA) to remove acid soluble material.

II. The lipid was extracted by organic solvents.

III. The residue formed the third group.

The whole sample as well as the three groups of compounds were then subjected to both qualitative and quantitative analysis.

Procedure.—One ml of a L.G. suspension containing 150–200 mg of dry substance was extracted with 2.5 ml of cold 10 per cent TCA. It was then centrifuged and the precipitate re-suspended in TCA. This process was repeated twice. Further extractions gave only extremely small additional amounts. The two extracts formed the acid soluble fraction (I). — The precipitate was first washed at room temperature with 5 ml of 50 per cent ethanol and then with 5 ml of 96 per cent ethanol to remove all TCA. The residue was boiled for 5 minutes with 5-ml portions of a 3 : 1 mixture of ethanol and ether. This procedure was performed 3 times. These extracts together constituted the lipoidal fraction (II). The residue (III) was essentially made up of proteins and nucleic acids.

The three main fractions were analyzed for phosphorus according to King [14]. The nitrogen content was determined by micro-Kjeldahl analysis and the sulfur content by a conductometric method according to Paulsson [19].

For the dry weight determination, an aliquot of the suspension was dried to constant weight at 100°C. In some cases the sample was dehydrated by freeze-drying. In all cases the value was corrected for the calculated amount of salt or sucrose in the suspension.

Fraction (I) was qualitatively analyzed with paper chromatography in butanol-acetic acid and water or phenol water for amino acids, sugars, and phosphate esters. In some cases attempts were made to analyze the fraction according to G. A. Le Page [25].

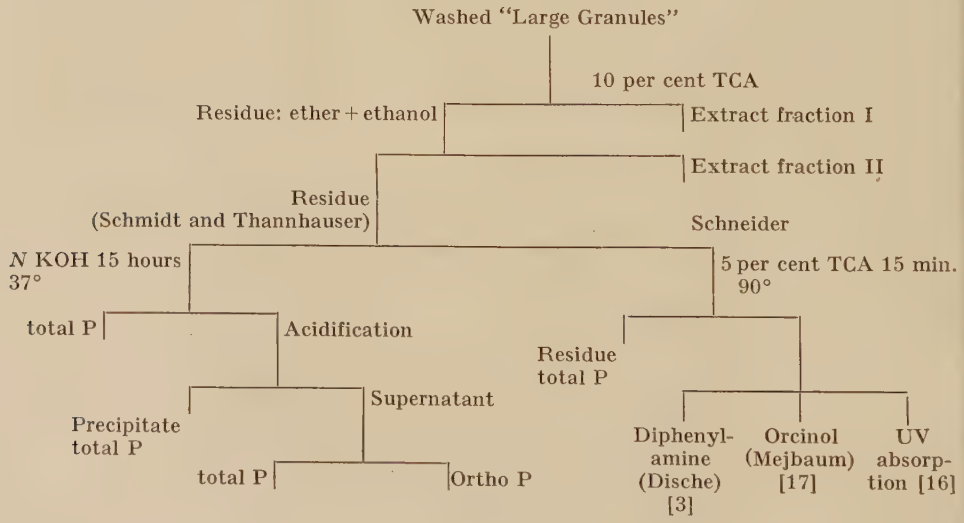


Fig. 3. Schematic outline of determinations.

The lipid fraction (II) was evaporated and dissolved in chloroform. The chloroform extract was dried at 50° and weighed. For the analysis of fraction (III) methods given by Schneider [22] and also by Schmidt and Thannhauser [21], were used in a modified form.

According to Schmidt and Thannhauser, the fraction was hydrolyzed for 15 hours at 37° in *N* KOH. The hydrolysate was divided into two portions, one of which was used for the determination of total phosphorus content, the other for precipitation of the DNA with 5 per cent TCA. The supernatant liquor was used for the determination of total phosphorus and ortho phosphorus compounds, according to Ernster *et al.* [7].

According to Schneider, another part of fraction III was treated with 5 per cent TCA for 15 minutes at 90°. Portions of the extract were used for the determination of DNA by the diphenylamine reaction of Dische [3] and of RNA by the orcinol method according to Mejbaum [17]. One aliquot part was analyzed by ultraviolet absorption methods [16, 26].

RESULTS

General Composition of Mast Cell Large Granules

Average values[1] expressed in per cent of the dry weight

	Acid soluble fraction (I)	Lipid fraction (II)	Protein fraction (III)	Total amount
Nitrogen	1.04	0.40	11.6	13.0
Phosphorus	0.13	0.82	0.40	1.40
Sulfur	0.09	Less than 0.01	0.29	0.39
Proteins and nucleoproteins				72.0
Lipids				23.9

[1] These values are the averages of six or eight determinations, made by the same procedures and under the same conditions.

Composition of the Various Fractions

Acid soluble fraction

	Result	Method
Amino acids	Leucine, glutamic acid tyrosine, glycine, histidine	Paper chromatography
Carbohydrates	Glucose, ribose, glucosamine Two unidentified substances R_F values in butanol 0.36, 0.24	Paper chromatography

	Result	Method
Phosphorus containing subst.	Inorganic P Glucose (1) phosphate Pyruvic phosphate	Paper chromatography by Hanes and Isherwood [8]
Miscellaneous components	Cytochrome C Nicotinamide Adenylic acid, histamine	Spectrophotometry Umbreit *et al.* [25]

Lipid fraction

This part constituted 23.9 per cent of the total dry weight. Elementary analysis gave 1.68 per cent nitrogen and 3.45 per cent phosphorus. The main part of the lipid fraction was not soluble in acetone which indicated a high percentage of lecithin. The constituent ratio of lecithin to phosphorus 25:1 gave a calculated lecithin content of 86 per cent.

The ratio of phosphorus to nitrogen (0.9) seemed to indicate the presence of some lipid rich in nitrogen. Small amounts of cholesterol were present. Amino acids and carbohydrates were not detected.

Residue

From the nitrogen determinations it was possible to make an approximate estimation of the protein content by using the formula: $6.25 \times \% \text{ N} = \%$ Protein. This yielded a value of 72 per cent protein in the large granules.

Nucleic acid determination: The method of Schmidt and Thannhauser [21] gave traces of DNA phosphorus. The amount of RNA-phosphorus was 0.36 per cent which corresponds to 3.6 per cent RNA. Schneider's method [22] showed no DNA with diphenylamine. Orcinol reagent [17] gave sugar values corresponding to 16.2 per cent RNA. A paper chromatogram, however, showed the presence of both glucose and ribose. Determination of ribose (in the presence of other sugars) according to Dische [3] gave results corresponding to 4–6 per cent RNA. Calculations from the purine-pyrimidine content by ultra violet absorption according to Logan *et al.* [16] gave 3.10 per cent RNA.

After hydrolysis of the residue and subsequent paper electrophoresis and chromatography the following components were found.

Amino-acids: Tyrosine, threonine, glutamic acid, glycine, alanine, aspartic acid, serine, leucine, valine, isoleucine, lysine, phenylalanine, cystine and methionine.

Carbohydrates: Glucose, ribose, glucosamine, and probably hexuronic acid.
Purines and pyrimidines: Adenine, guanine, cytosine and uracil.

Determination of Anticoagulating Effect

The most effective means for extracting heparin was devised by Wilander [27]. It involves treating the washed L.G. with $(NH_4)_2SO_4$ and NaOH. By this method the yield of heparin was less than 0.3 μg per g of washed L.G. One g of fresh ox-liver capsule treated in the same way gave 450 μg of heparin.

The amounts of heparin were determined by means of a biological blood clotting method according to Jaques and Charles [11].

DISCUSSION

The isolation and subsequent analysis of the different parts of cells by differential centrifugation of tissue homogenates is a widely used technique which previously has been applied to studies on liver, kidney, muscle and other tissue, compare [25, 5]. The chemical composition of nuclei, mitochondria and microsomes, within the fractions so obtained has been reported, as has also the presence of certain enzymes and enzyme systems.

However, as concerns investigations of connective tissue the differential centrifugation method has been less widely used because of the difficulty encountered in obtaining a suitable homogenate from this material. Nevertheless, with this technique a considerable measure of success was achieved in the present investigation. Fresh ox-liver capsules were used as raw material from which mast cell granules were isolated. Such material was specifically chosen since it constituted an extremely rich source of mast cells and, further, other cells present, principally fibroblasts, contained granules of a comparatively small size, thus facilitating the preparation of a remarkably well defined and uniform fraction in contrast to that obtained from liver.

In order to avoid the precipitation of heparin on the surface of the large granules during the isolation process, it was essential to observe the following precautions: It was necessary to suspend the homogenate in either a sugar or a salt solution and to control the pH of the final mixture very accurately. The divergent results published by Zollinger [28] and especially by Köksal [15], in which they report the observation of heparin in these granules, may be due to the disregard of these two essential precautions.

The chemical composition of washed large granules showed a remarkable similarity to that of mitochondria from other tissue. Swanson and Artom [24], for instance, have reported the following analysis for liver mitochondria (values in per cent): N 12.12, P 1.38, protein 63.4, lipids 22.45. Preliminary

enzyme investigations have shown that these large granules, like mitochondria, contain respiratory enzymes. This observation is supported by the chemical analysis which showed the presence of cytochrome c and nicotinamide.

Riley [20] has found a strong positive correlation between the number of mast cells and the histamine content of different tissues. The present investigation indicates that the histamine is connected with the granules of the mast cells.

SUMMARY

The large cytoplasmic granules of connective tissue mast cells have been isolated by a method of differential centrifugation. The chemical analysis of the selected washed granular fraction gave the following general composition (values in per cent): N 13.0, P 1.40, S. 0.39, proteins 72, lipids 23.9. Tests showed that anticoagulant properties were not present. Histamine was found to be connected with the granular fraction.

The costs have been defrayed by grants from the Swedish Anti-Cancer Society and the Cancer Research Division of Radiumhemmet.

REFERENCES

1. Asboe-Hansen, G., *Intern. Rev. Cytol.* **III**, 399 (1954).
2. Dalgaard, J. B., *Ugeskrift for Læger* **18**, 513 (1948).
3. Dische, Z., *Mikrochemie* **8**, 4 (1930).
4. Dounce, A. L., *Cancer Research* **11**, 562 (1951).
5. de Duve, C. and Berthet, J., *Intern. Rev. Cytol.* **III**, 225 (1954).
6. Ehrlich, P., *Arch. mikrosk. Anat. Forsch.* **3**, 263 (1877).
7. Ernster, L., Zetterström, R., and Lindberg, O., *Acta chem. scand.* **4**, 942 (1950).
8. Hanes, C. S. and Isherwood, F. A., *Nature* **164**, 1107 (1949).
9. Hogeboom, G. H., Schneider, W. C., and Palade, C. E., *J. Biol. Chem.* **172**, 619 (1948).
10. Holmgren, H. and Wilander, O., *Z. Mikroskop. Anat. Forsch.* **42**, 242 (1937).
11. Jaques, L. B. and Charles, A. F., *Quart. J. Pharm. and Pharmacol.* **14**, 1 (1941).
12. Jorpes, J. E., Heparin in the Treatment of Trombosis. London. Oxford University Press, pp. 62–63, 1946.
13. Julén, C., Snellman, O., and Sylvén, B., *Acta physiol. Scand.* **19**, 289 (1950).
14. King, E. J., *Biochem. J.* **26**, 292 (1932).
15. Köksal, M., *Nature* **172**, 733 (1953).
16. Logan, J. E., Mannell, W. A., and Rositer, R. J., *Biochem. J.* **51**, 480 (1952).
17. Mejbaum, W., *Hoppe-Seyler's Z. physiol. Chem.* **258**, 117 (1939).
18. Noback, C. R. and Montagna, W., *Anat. Rec.* **96**, 279 (1947).
19. Paulsson, S., *Acta Chem. Scand.* **7**, 325 (1953).
20. Riley, J. F. and West, G. B., *J. Physiol.* **120**, 522 (1953).
21. Schmidt, G. and Thannhauser, S. J., *J. Biol. Chem.* **161**, 83 (1945).
22. Schneider, W., *ibid.* **161**, 293 (1945).
23. Schneider, W. C. and Hogeboom, G. H., *Cancer Research* **11**, 1 (1951).
24. Swanson, M. A. and Artom, C., *J. Biol. Chem.* **187**, 281 (1950).
25. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric Technique and Tissue Metabolism. Burgess Publishing Co. 1949, New York, 1949.
26. Vischer, E. and Chargaff, E., *J. Biol. Chem.* **176**, 715 (1948).
27. Wilander, O., *Scand. Arch. f. Physiol.* **81**, Suppl. No. 15 (1938).
28. Zollinger, H. U., *Experientia*, Basel, **6**, 384 (1950).

Experimental Cell Research, **9**, *157–167 (1955)*

INTERRUPTION OF THE CORTICAL REACTION BY HEAT

R. D. ALLEN[1, 2] and B. HAGSTRÖM

The Wenner-Grens Institute of Experimental Biology, University of Stockholm, Sweden

Received December 7, 1954

The fertilization impulse in the egg of the sea urchin is a change which displays itself within the range of submicroscopic dimensions. It is initiated at some time during sperm attachment and penetration, and propagated over the egg surface in about 20 seconds [2, 12, 14]. The first visible expression of this submicroscopic change is the breakdown of the cortical granules which follows immediately. The granule breakdown is in turn followed by elevation of the fertilization membrane, increase in the thickness of the hyaline layer, and a series of later changes also brought about by fertilization. Although much attention has been given to the genetic and cytological contributions of a sperm nucleus to the zygote, very little significance has been attached to the influence of the cortical reaction on cytoplasmic and nuclear changes which follow fertilization. Information concerning cortical changes at fertilization has been presented by one of us (R. D. A.) in a series of papers dealing with *partially fertilized eggs* produced by stretching the cell surface [2, 3, 4]. Examination of such eggs proved that the changes in structure associated with fertilization had occurred only on one side. The other side was completely unfertilized according to accepted criteria, and, furthermore, was receptive to reinsemination.

Partially fertilized eggs provide a unique situation for the examination of (1) the role of the cortical reaction in cytoplasmic and nuclear movements, cell division and later development, and (2) the interaction of the cortex and endoplasm. For this reason, a convenient method for the production of large numbers of partially fertilized eggs was sought in the present work.

Insemination at temperatures higher than normally encountered under natural conditions has been known to cause polyspermy [9]. It was a second purpose behind the present experiments to determine whether heat-induced polyspermy might have been caused by interruption of some phase of the

[1] Postdoctoral Fellow of the National Cancer Institute, National Institutes of Health, U.S. Public Health Service.

[2] Present address: Department of Zoology, University of Michigan, Ann Arbor, Michigan, U.S.A.

cortical reaction, in which case partially fertilized eggs would be obtained if a means were found to inactivate refertilizing spermatozoa. One of us (B. H.) has recently introduced a method for measuring the rate of fertilization by treating mixed gametes at fixed intervals during insemination with the detergent sodium laurylsulfate, which inactivates sea urchin spermatozoa on contact [6]. This fertilization rate technique was employed in the present study with the addition of heat as an experimental condition.

MATERIAL AND METHODS

Mature gametes of the following species of sea urchins were allowed to be shed from washed, excised gonads: *Psammechinus microtuberculatus*, *Paracentrotus lividus*, *Sphaerechinus granularis* and *Arbacia lixula* from the Bay of Naples; and *Psammechinus miliaris* from the Swedish west coast. Experiments were performed both with the jelly intact and on jelly-free eggs; no significant differences were encountered.

To obtain partial fertilization, a series of beakers, each containing 20 ml of 0.001 per cent sodium laurylsulfate in sea water, was placed in a bath containing warm water (37–38°C). When the beakers had reached a constant temperature, a sample of eggs was inseminated at 15–18°C with "normal sperm density" (about 3.5×10^6 sperm/ml); aliquots of the fertilizing gametes were then transferred with wide mouth pipettes at 10 second intervals to the warm sea water containing detergent. Mixtures of eggs and detergent were stirred for 20 seconds and then removed from the warm bath, divided into two portions and allowed to cool. Rapid cooling (by cold baths) was found to produce injury. However, if mixing was rapid and thorough, the eggs were not required to endure temperatures higher than 28–32°C. After two minutes of gradual cooling, fresh sea water was added; after settling, the eggs were washed by decantation followed by flooding with fresh sea water.

Observations were made with Leitz 22 × and 50 × water immersion objectives. Dark field illumination was provided by a Zeiss *Wechselkondensor nach Siedentopf* (with oil between condensor and slide).

RESULTS

Insemination at high temperatures; pre- and post-fertilization heating.— Insemination was carried out at temperatures from 18–35°C in vessels suspended in a constant temperature bath. At normal sperm concentrations polyspermy began to appear above 27°C and became widespread above 30°. At these temperatures and higher it was clear that the cortical reaction was being interfered with, as membrane elevation was abnormal or lacking. Sperm were able to penetrate, however at temperatures as high as 35°.

Unfertilized eggs were exposed to temperatures up to 33°C for one minute and then inseminated after cooling to room temperature, with normal sperm concentrations. No polyspermy was observed, and development was

normal. Exposure to 33°C two minutes after fertilization for an interval of 20 seconds had no deleterious effect on development, and no polyspermic development was observed following attempted reinsemination.

Insemination at room temperature followed by treatment with warm detergent solutions.—Lots of eggs which had been inseminated at room temperature and

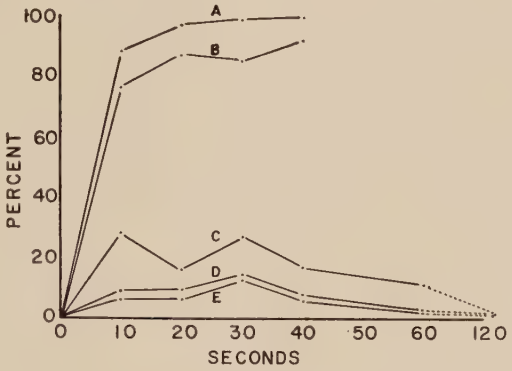


Fig. 1. The results of a typical experiment to produce partial fertilization. *A*, percentage of total activation; *B*, percentage of normal cleavage; *C*, percentage of polyspermy on refertilization; *D*, percentage partially fertilized eggs; and *E*, percentage of partially fertilized eggs which cleaved. The abscissa designates the times at which heat was applied (see methods).

immediately exposed to detergent solutions (final temperature 28-32°C) were examined for partially fertilized eggs. Partially fertilized eggs of *P. lividus* were especially easy to identify; they almost always possessed a bulge on the fertilized side, or had a pear-shaped outline. The eggs of *A. lixula* showed this shape characteristic to a diminished degree. *S. granularis* and both species of *Psammechinus* exhibited less change in shape and were therefore less easily recognized when partially fertilized. Consequently, *Paracentrotus lividus* eggs were used for most of the experiments, and the results were checked with the other species mentioned.

The results of a typical experiment are presented in Fig. 1. The percentage of total activation is the same as the fertilization rate [cf. 7]. The difference between the percentage total activation and the percentage normal cleavage is accounted for by the percentage of partially fertilized eggs. All eggs showing pear-shaped outline or intact cortical granules on any part of the surface were counted as partially fertilized. It is clear from the percentage polyspermy on refertilization that the block to polyspermy [cf. 11] had not been established in some of the eggs which had shown complete cortical reactions. It was noted that the increase in thickness of the hyaline layer which occurs after passage of the fertilization impulse and breakdown of the cortical granules could be partly inhibited by the application of heat less than two minutes after passage of the cortical change. It is probable that this damage

to the hyaline layer was responsible for the percentage of polyspermy on refertilization in excess of the percentage of partially fertilized eggs. In some experiments the percentage of refertilization was slightly less than the percentage of partially fertilized eggs. However, it must be kept in mind that normal to light sperm concentrations were used in refertilization.

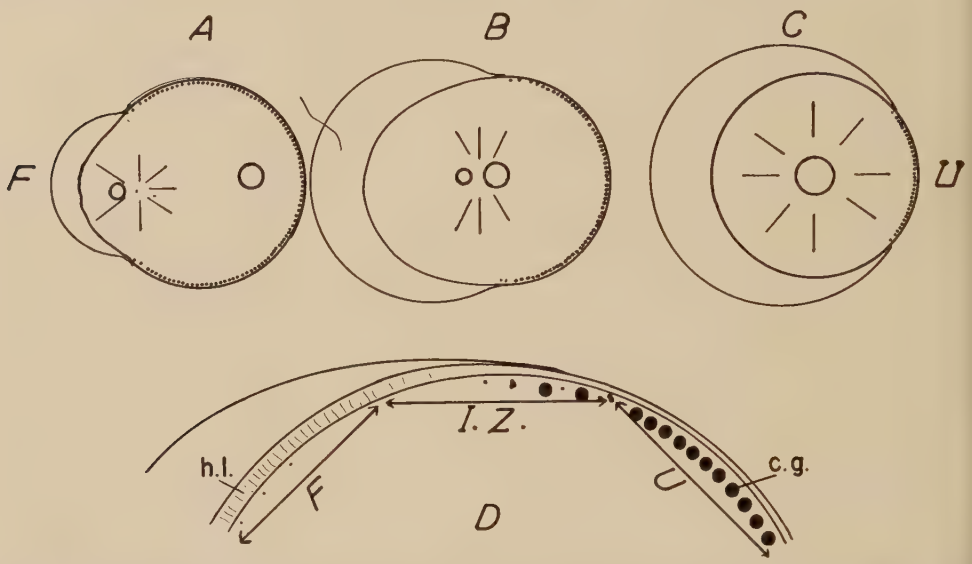


Fig. 2. A diagram to show different degrees of partial fertilization in three different eggs. *A*, less than 50 per cent of the surface affected by the fertilization impulse; *B*, more than 50 per cent; *C*, only a few cortical granules remaining. *D*, an enlarged view of the intermediate zone. (*h.l.* = hyaline layer, *c.g.* = cortical granule, *i.z.* = intermediate zone, *F* = fertilized zone, *U* = unfertilized zone.)

The surface structure of partially fertilized eggs.—Various degrees of partial fertilization were observed (see Fig. 2). At the point of fertilization (*F*), the fertilization membrane was always most highly elevated, and all cortical granules were broken down. At the pole of the egg opposite fertilization (*U*), the cortical granules were completely intact and no membrane was visible. Scanning the surface from (*F*) to (*U*), the fertilization membrane became progressively lower, then absent; the hyaline layer became thinner, and the number of cortical granules per unit of surface area increased. This zone of transition between the fertilized and unfertilized zones has been called the *intermediate zone* [2, 3]. The width of this zone was dependent upon the temperature of the detergent-egg mixture. Temperatures from 29–31°C produced the sharpest zone differentiation without deleterious effects on development.

Eggs with a wide intermediate zone showed a gradient with an inverse relationship between the number of cortical granules present and the thickness of the hyaline layer. Eggs with narrow intermediate zones showed steeper gradients. Since the hyaline layer could be followed from the fertilized zone over the intermediate zone to the unfertilized zone, it was concluded that a thin hyaline layer was present in the unfertilized egg. Comparisons with untreated, unfertilized eggs confirmed this conclusion.

In most partially fertilized eggs, the wave of contraction which normally accompanied cortical granule breakdown became "fixed" in the intermediate zone in the form of a wrinkling which sometimes vanished during cleavage.

Partially fertilized eggs exhibited two clearly-defined color zones when viewed under dark-field illumination. The fertilized zone was the silvery-white color encountered in fertilized eggs; the unfertilized zone was orange-yellow [cf. 13].

Several partially fertilized eggs were refertilized under the microscope. Membranes were seen to become elevated, and the remaining cortical granules broke down on what had been the unfertilized side. By means of aceto-carmine staining, it was confirmed that sperm penetration had been on the unfertilized side. Refertilized partially fertilized eggs cleaved in the manner expected of polyspermic eggs. Only light insemination was required for refertilization; no delay or difficulty was encountered, even as late as the four-cell stage.

A spontaneous breakdown of the cortical granules in the unfertilized zone was occasionally observed 10–30 minutes after fertilization. This breakdown was clearly not caused by entrance of another sperm, or to a resumption of the fertilization impulse; it more closely resembled the slow breakdown following some kinds of artificial activation [4]. A rapid increase in the thickness of the hyaline layer followed this gradual cortical change. This phenomenon occurred occasionally in the eggs of both species of *Psammechinus,* and rarely in those of *P. lividus.*

Nuclear migration in partially fertilized eggs.—Interruption of the cortical reaction was found to impede subsequent movements of the egg and sperm nuclei. The penetration path of the sperm nucleus was normal if at least 40–50 per cent of the cortex had been converted by the cortical reaction. A reduction from this value resulted in stunted asters and shortened penetration paths. In cases where the cortical reaction had been stopped after only 10–20 per cent of the cortex had been converted, the sperm head came to lie just beneath the egg surface, and apparently no aster formed.

Activation and migration of the egg nucleus depended not only upon the

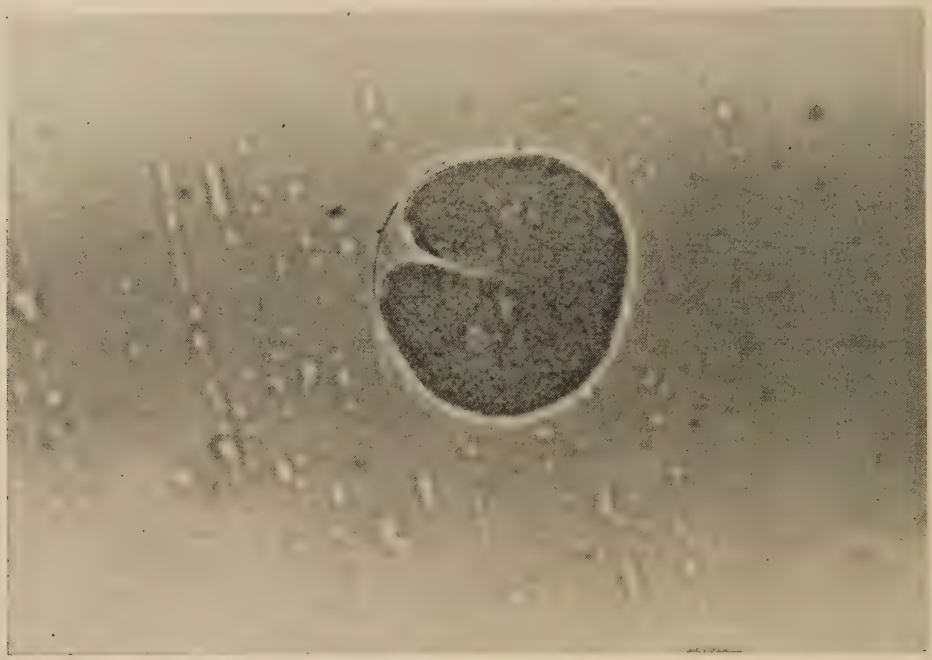


Fig. 3. A photograph of an incomplete cleavage furrow in a partially fertilized *Psammechinus* egg.

amount of cortical material converted, but also upon the distance of the egg nucleus from this fertilized cortex. In partially fertilized eggs with the sperm and egg nuclei separated at different poles of the egg, there was a greater tendency for failure of nuclear activation, migration and fusion. For this reason, eggs fertilized in the animal hemisphere had a better chance to cleave and develop. In various experiments, up to 30 per cent of the partially fertilized eggs failed to show nuclear fusion; most of these eggs were vegetally fertilized, and among them were all degrees of abortive nuclear activation and migration (cf. Fig. 2).

Cleavage delay and developmental abnormalities of partially fertilized eggs.— Partially fertilized eggs which did not exhibit nuclear fusion failed to divide. Mitotic spindles were lacking entirely. Among those eggs showing nuclear fusion, there was a cleavage delay of up to 20 minutes for the first division. This delay was clearly correlated with the amount of unfertilized cortex present in the egg. The cleavage furrow always began on the fertilized hemisphere and progressed slowly. Often this furrow failed to divide the egg completely (cf. Fig. 3). Those partially fertilized eggs which cleaved were easily discernible from normal 2-cell stages because they had retained their

pear shape, and often one blastomere was smaller or larger than the other. If the first cleavage divided the fertilized and unfertilized hemispheres, it was sometimes seen that the cells in the fertilized hemisphere assumed a more rapid mitotic rhythm. The results of experiments on *Arbacia lixula* were distinguished by two features: (1) the fertilized and unfertilized hemispheres were different shades of red; this apparently depended upon the fact that migration of the pigment granules had taken place only on the fertilized side. (2) Either for this or for some other reason, *Arbacia* eggs failed to develop further than the 2–4 cell stages in marked contrast to the partially fertilized eggs of the other species studied.

The poor development of the hyaline layer in most partially fertilized eggs usually resulted in the formation of cell plates after several cleavages. This was apparently immediately caused by failure of some cementing substance usually found in the hyaline layer. Those eggs which managed to keep their blastomeres together developed into 'filled blastulae'' or into animalized larvae with short arms, reduced gut, etc. Very few larvae lived long enough to exhibit interesting abnormalities.

DISCUSSION

Conversion of the egg surface from the unfertilized to the fertilized condition is now thought to include several related processes or steps. Sperm attachment initiates the first of these processes: the fertilization impulse [2, 3, 15] (Sugiyama's ''fertilization wave''). This impulse is in itself apparently not visibly manifested; its first visible expression is the breakdown of the cortical granules. The latter takes about 20 seconds and is followed by the elevation of the fertilization membrane [12]. During this time there is a gradual increase in the thickness of the hyaline layer to a maximum at about 1 minute after sperm attachment. At about this time, the block to polyspermy is complete [13].

The series of processes outlined above constitutes the ''cortical reaction''. The problem arises: what specific process or processes are interrupted by the action of heat? If heat had no effect on the fertilization impulse, but rather inhibited cortical granule breakdown directly, it would be expected that whatever initial change was brought about by the fertilization impulse would remain for 20 seconds (the presentation time for heat), and then elicit the subsequent steps in the fertilization reaction when the temperature dropped. No resumption of the cortical reaction was observed, nor was there any indication that a change had taken place in the unfertilized zone,

either through the action of heat or from the passage of any fertilization change. Furthermore, there is no reason to suspect that heat would inhibit cortical granule breakdown, for high temperatures themselves can under certain circumstances be stimuli for cortical granule breakdown. In fact, a few eggs were observed in the present study which were apparently "partially artificially activated"; that is, eggs without sperm nuclei that would otherwise have been classified as partially fertilized. Such activation might have been brought about by sperm which attached in such a way as to initiate the cortical reaction, but failed to penetrate, or, it may have been the result of activation by high temperature. It is concluded that heat most probably inhibits the cortical reaction by interrupting the fertilization impulse.

Previous experiments employing the capillary technique had led to the conclusion that the fertilization impulse was a chain reaction [cf. 14] between some kind of submicroscopic structural "units" [3, 14], the exact nature of which is unknown. Partially fertilized eggs were obtained in these earlier studies by stretching the egg surface. Presumably, stretching brought these units further apart, and, therefore, progressively weakened the impulse. It is logical to assume that molecular motion at higher temperatures would have the same disrupting effect on fine structure as stretching. Unpublished capillary experiments (R. D. A.) have indicated that the effect of stretching could have been enhanced by slightly elevated temperatures and completely abolished by slightly lowered temperatures. At 3–12°C it was found impossible to inhibit the fertilization impulse by surface stretching. A similar general temperature effect was found on the artificial activation of surf-clam eggs [1].

Since hyaline layer formation takes place after the underlying cortical granules have broken down, the question arises as to whether hyaline layer growth (1) depends upon prior passage of the fertilization impulse, or (2) depends upon prior disappearance of the cortical granules and not necessarily upon the fertilization impulse. The only evidence available on this problem comes from (1) the spontaneous cortical granule breakdown sometimes observed 10–30 minutes after fertilization in the unfertilized zone. This breakdown did not have the character of breakdown induced by the fertilization impulse, and yet it was followed rapidly by growth of a hyaline layer; (2) it has been observed elsewhere (unpublished observation) that some artificial activating agents (hyper- and hypotonic sea water) which do not initiate a fertilization impulse cause an increase in the thickness of the hyaline layer following cortical granule breakdown. It seems likely from this evidence that it is cortical granule breakdown and not necessarily the fertilization impulse, which permits the normal thickening of the hyaline layer.

Experiments performed by one of us (B. H.) suggested that the formation of a normal hyaline layer is essential in preventing polyspermy. Treatments which remove the hyaline layer or damage it permit easy refertilization [7, 8]. Since it is known that the block to polyspermy is not complete until about the same time that the hyaline layer formation is complete, it is tempting to speculate that the growth of the hyaline layer may account for establishment of the slow, complete block to polyspermy [cf. 12]. Rothschild has proposed the existence of a rapid, incomplete block to polyspermy in addition to the slow, incomplete block. However, the extreme ease of refertilization of partially fertilized eggs indicated that no irreversible rapid block to polyspermy occurred before passage of the fertilization impulse.

A clear relationship has never been demonstrated between the cortical reaction and later events, such as cleavage and development. Although activation always involves conversion of the cortex from unfertilized to fertilized condition, the cortical reaction has always been considered as of secondary importance. It is now becoming clear that the cortical reaction is a necessary step in the activation of the main mass of egg protoplasm in preparation for nuclear activation and fusion, and for cell division and development. Growth of the sperm aster sends the sperm nucleus on a path perpendicular to any tangent to the egg surface (penetration path). It has been previously observed that sperm fail to form asters when they enter through injured cortex [14]. Thus the opinion has been expressed that aster formation and growth may depend upon a reaction between the sperm and the cortex of the egg [3, 14]. This contention has been further supported by the observation that restriction of the amount of fertilized cortex around the point of sperm penetration limits the growth of the sperm aster and consequently the distance of penetration of the sperm nucleus. An alternative possibility is that the cortex after fertilization converts a limited amount of underlying cytoplasm into a new fertilized condition, and that this condition is essential for the establishment of an aster. The behaviour of the egg nucleus has given some support to the latter view. The egg nucleus failed to respond to the presence of the sperm if either nucleus was largely surrounded by unfertilized cortical material. Partially fertilized eggs frequently exhibited quite dissimilar light-scattering properties on the fertilized and unfertilized sides; this difference was more marked in those eggs which failed to divide. It seems possible that this failure to divide may have been in part caused by failure of the fertilized and unfertilized cytoplasm to mix. Such mixing usually does not occur normally in partially fertilized eggs obtained by the capillary method (R. D. A., unpublished results). These eggs also rarely

cleave on their unfertilized parts, and light scattering differences are particularly pronounced.

The question of developmental abnormalities caused by the presence of unfertilized cortex requires much more study. The commonest abnormality, the formation of "cell plates", is probably caused by the poor development of the hyaline layer on regions containing remains of the cortical granules. Poor hyaline layer development resulted in faulty cementing of the blastomeres of partially fertilized embryos. The various degrees of animalization observed in the few larvae developing from partially fertilized eggs have been attributed to a decreased inducing influence from the vegetal region of these eggs [10]. It is believed that the opposite condition (i.e. vegetalization) did not occur because of the failure of vegetally fertilized eggs to undergo nuclear fusion and cleavage.

SUMMARY

The fertilization impulse and associated structural changes accompanying the cortical response to sperm attachment in the sea urchin egg were interrupted within 20 seconds after sperm attachment by a 20 second exposure to warm sea water containing small amounts of the detergent sodium laurylsulfate (to prevent entry of a second sperm). Substantial numbers of partially fertilized eggs were isolated and observed for surface structure, nuclear movements, cleavage details and rate, and later development. Partially fertilized eggs showed a *fertilized zone*, an *unfertilized zone* and an *intermediate zone*. The unfertilized zone remained freely penetrable to refertilizing spermatozoa, showing that no irreversible block to polyspermy had preceeded the cortical reaction. The intermediate zone exhibited a gradient of hyaline layer thickness inversely related to a gradient of cortical granule number. From this and other evidence, it was concluded that the increase in hyaline layer thickness depended upon prior breakdown of the cortical granules. It was suggested that the hyaline layer might play and important role in the final establishment of the block to polyspermy, because thin hyaline layers could be penetrated by refertilizing spermatozoa.

The presence of unfertilized cortex in partially fertilized eggs was found to retard or arrest some of the processes associated with early and late development. Various degrees of inhibition of nuclear movements were correlated with corresponding degrees of partial fertilization. Eggs which exhibited nuclear fusion usually cleaved, and in such cases the cleavage furrow began to form on the side nearest the point of sperm entry. Increasing

amounts of unfertilized cortex were found to cause more delay in cell division.

The development of most partially fertilized eggs led to a formation of "cell plates" because of the failure of hyaline layer growth and cement formation. The embryos the cells of which held together usually formed animalized larvae, for eggs fertilized on the animal hemisphere had a better chance to undergo nuclear fusion.

We are both indebted to Professor John Runnström for his active interest in this work and for his advice and suggestions. We wish also to thank the officials of the Stazione Zoologica, Naples, Italy, and the Kristinebergs Zoological Station, Fiskebäckskil, Sweden for their kind hospitality.

REFERENCES

1. Allen, R. D., *Biol. Bull.* **105**, 213 (1953).
2. —— *Exptl. Cell Research* **6**, 403 (1954).
3. —— *ibid.* **6**, 412 (1954).
4. —— *ibid.* **6**, 422 (1954).
5. Chambers, E. L., *J. Exptl. Biol.* **16**, 409 (1939).
6. Hagström, B. and Hagström, Britt, *Exptl. Cell Research* **6**, 479 (1954).
7. —— *ibid.* **6**, 491 (1954).
8. —— *ibid.* **6**, 532 (1954).
9. Hertwig, O. and R., *Jen. Zeitsch.* **20**, 120 (1887).
10. Hörstadius, S., *Biol. Revs.* **14**, 132 (1939).
11. Rothschild, Lord, *ibid.* **30** (1), 57 (1953).
12. Rothschild, Lord and Swann, M. M., *J. Exptl. Biol.* **26** (2), 164 (1949).
13. Runnström, J., *Acta Zool.* **4**, 285 (1923).
14. Runnström, J. and Kriszat, G., *Exptl. Cell Research* **3**, 419 (1952).
15. Sugiyama, M., *Biol. Bull.* **104**, 216 (1953).

ON THE RESISTANCE OF NORMAL AND MALIGNANT TISSUE CELLS *IN VITRO*

A. FISCHER

Biological Institute of the Carlsberg Foundation, Copenhagen, Denmark

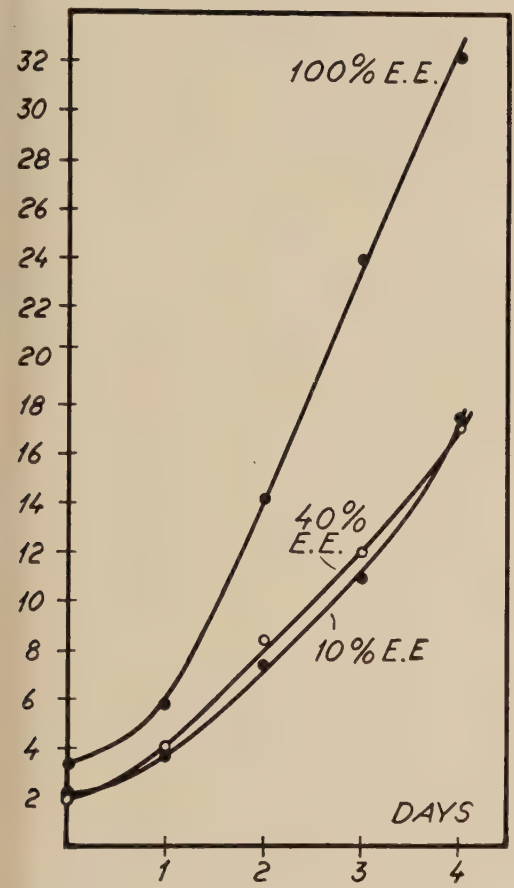
Received December 20, 1954

Tissue cells grown *in vitro* cannot be identified in tissue cultures—on account of the dedifferentiation taking place during their cultivation. It is possible only grossly to distinguish morphologically between epithelial and mesenchymal tissues *in vitro*. It has proved possible, however, to differentiate between heart fibroblasts and osteoblasts, chondroblasts and skeletal muscle fibroblasts with a technique described by A Fischer [2]. The method consisted in growing the respective tissue cells in the presence of various amounts of embryo extract and measuring the area of the culture as a function of the embryo extract concentration which graphically gave different curves, each characteristic of its own type of tissue. The growth of osteoblasts and chondroblasts was inhibited by excess of embryo extract.

By means of this method we have now tried to differentiate between heart fibroblasts from 9-day-old embryos and malignant tissue (fibrosarcoma produced by injection of methylcholanthrene into the breast musculature on a hen). This proved impossible, since the curves obtained were identical (Figs. 1 and 2).

In order to compare tumour tissue and normal tissue we also investigated the stability of the cells in tissue cultures. First we studied how long tissue fragments—as far as possible of the same size—and tissue cultures remained viable after being kept in Ringer's solution at 2°C. The viability was determined in this way: At different time intervals uniform pieces of tissue (about 2×2 mm) or cultures were placed in a normal plasma medium. The viability for the tumour tissue was found to amount to four days while for the normal heart tissue it was six days.

We employed also another method for comparison between methylcholanthrene tumour tissue and embryonal heart tissue. It seemed desirable to determine the point of time for plasmolysis. Similarly Lucke and Parpart [3] compared the permeability of cancer cells with that of erythrocytes. We therefore placed tissue fragments as well as tissue cultures in redistilled water at 38°C, and at time intervals from 5 min. to 50 min. pieces of tissue or cultures



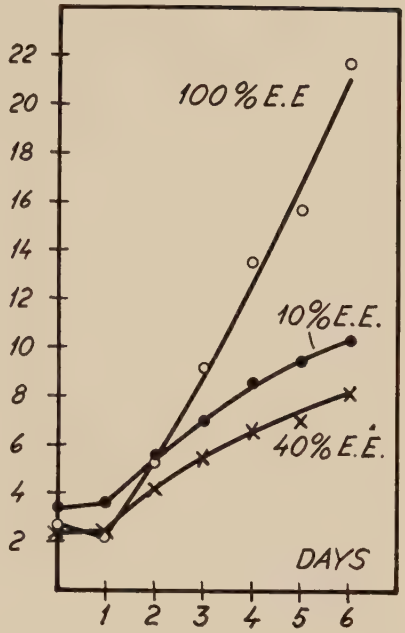


Fig. 1 (left). Growth of heart fibroblasts in the presence of different concentrations of embryo extract (EE). *Abscissa:* Days; *Ordinate:* Growth area.
Fig. 2 (above). Growth of tissue from a methylcolanthrene tumour *in vitro* in the presence of different concentrations of embryo extract (EE). Compare Fig. 1.

were transferred to normal media in order to ascertain their viability under these conditions. The limit for the viability of the tumour tissue was found to be 35 min., while the heart tissue was able to start growing again after 50 min., i.e., it proved more resistant.

The stability of the two cell types has also been studied in other ways.

Heart fibroblasts and methylcholanthrene tumour cells were grown through several passages in which the cultures every three days were supplied with a fluid phase consisting of 0.2 ml horse serum, 0.2 ml Tyrode's solution and 0.1 ml highly diluted embryo extract. All the cultures of both cell types showed a profuse and dense growth of normal cells.

We then prolonged the interval between the passages—that is, the cultures were allowed to grow in the same glass without renewal of the fluid phase

until it looked as if they commenced to decay. Now the question was to decide whether the cells were living and able to regenerate, i.e., whether they were viable and again might be induced to proliferate. After intervals varying from 7 days to 3 months, the same picture was observed again, namely, that the tumour tissue after transfer only slowly began to grow again but then kept alive for a very long time. The heart fibroblasts commenced to grow at once and died out rather rapidly.

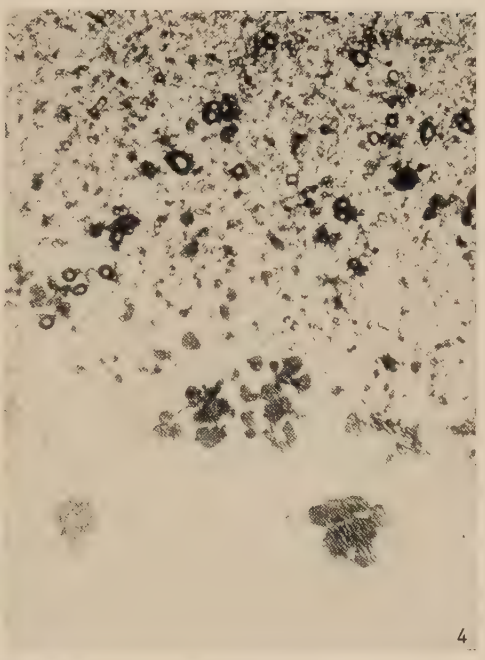
We used partly 1 ml plasma or 0.5 ml plasma in Carrel flasks and partly a monolayer of plasma [1]. These experiments indicate a regeneration *in situ*, i.e., the cells grow at the expense of decaying parts of the tissue—a state of stationary growth. Figs. 3 and 4 show sections from cultures of the two tissues employed after cultivation for 52 days in normal media in Carrel flasks, without changing the fluid phase. Figs. 5 and 6 show the same cultures after transplantation to new flasks.

As tissue cultures are able to grow abundantly on a very thin layer of normal plasma medium [1], we have performed the same experiments on monolayers of media dialysed for 2×2 days. Figs. 7 and 8 show a heart fibroblast culture and a culture of methylcholanthrene-induced tumour grown on a monolayer of dialysed plasma without compensation or change of fluid phase for 28 days. At this point of time the cultures looked as if they had died long ago, and they showed numerous large vacuoles. Figs. 9 and 10 show the same cultures after transfer to new flasks and in normal medium consisting of 1 ml chicken plasma, 0.5 ml Tyrode's solution and 0.1 ml embryo extract. It will be noticed that the cultures here present the appearance of perfectly normal cells.

Heart fibroblasts were grown in the same flask until three months on a monolayer of dialysed plasma without compensation and with 0.5 ml Tyrode's solution and 0.2 ml dialysed embryo extract for liquid phase. During these three months the liquid phase was not changed and the morphology of the cells suggested that they had perished long ago. The cultures were then transferred to new flasks containing a normal medium in order to see whether the cells might still be living. This was indeed the case as the cultures showed distinctly a lively growth though of not quite normal cells.

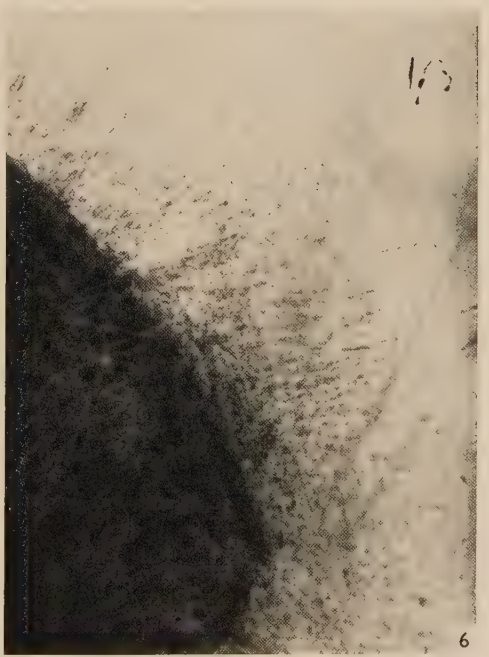
Fig. 3. Fibroblasts in normal media after 52 days without change of liquid phase.
Fig. 4. Methylcholanthrene tumour cells. (52 days in normal media without change of the liquid phase.)
Fig. 5. Heart fibroblasts. (In normal media for 52 days without change of the liquid phase. After transfer to new flasks.)
Fig. 6. Methylcholanthrene tumour. (52 days without change of the liquid phase. After transfer to new flasks.)

3

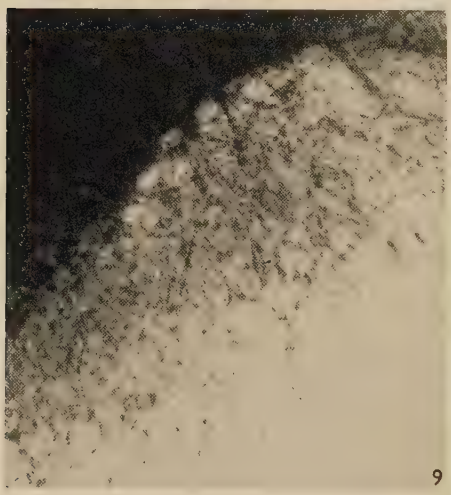


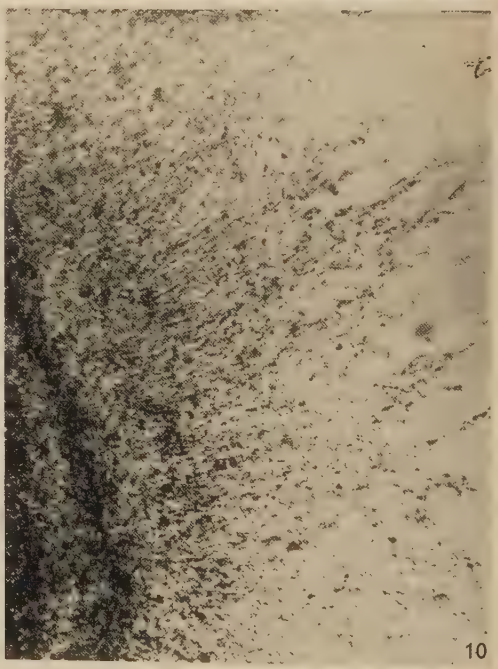
4

5



6





On continued cultivation of these cells in normal media with change of the liquid phase every other day the condition of the cells improved and approached the normal morphological picture.

In these comparative studies on the two kinds of tissues the feature common to the cultures is the fact that the tumour tissue (produced by injection of methylcholanthrene into the breast musculature of chicken) is very resistant and stable, keeping surprisingly well on a poor medium. The embryonal heart tissue here employed is considerably more labile and does not survive well under unfavourable conditions.

It is not always possible from the morphological picture to decide whether the cells in a given culture are living or dead.

This work was aided by a research grant from the National Cancer Institute of the National Institutes of Health, Public Health Service, U.S.A.

REFERENCES

1. Fischer, A., *Exptl. Cell Research* **6**, 523 (1954).
2. —— Gewebezüchtung. Verlag Müller & Steinicke, München. p. 209, 1930.
3. Lucke, E. and Parpart, A. K., *Cancer Research* **14**, 75 (1954).

Fig. 7. Heart fibroblasts, 27 days in dialysed media without change of the liquid phase.
Fig. 8. Methylcholanthrene tumour cells in dialysed media for 27 days without change of liquid phase.
Fig. 9. Heart fibroblasts in dialysed media for 27 days without change of liquid phase. (After transfer.)
Fig. 10. Methylcholanthrene tumour cells in dialysed media. (27 days without change of the liquid phase.) (After transfer.)

A GRADED RESPONSE TO ELECTRICAL CURRENT IN THE CORTEX OF THE SEA URCHIN EGG

R. D. ALLEN,[1,2] A. LUNDBERG and J. RUNNSTRÖM

Wenner-Grens Institute for Experimental Biology, University of Stockholm, and the Institute of Physiology, Lund, Sweden

Received January 18, 1955

The fertilization reaction is now believed to consist of a series of cortical changes with the character of step processes. Attachment of the sperm initiates the *fertilization impulse*, which spreads over the surface of the egg probably as a self-propagating chain reaction [1, 9]. The passage of the fertilization impulse is detected by the subsequent breakdown of the cortical granules in a wave, the character of which has suggested that these cortical granules may have a random assortment of individual thresholds [1]. The cortical granules are expelled into the perivitelline space and they are apparently involved in the formation of the fertilization membrane [2, 6, 8]. After the cortical granules have left the cortex, the hyaline layer, which is already present in the unfertilized egg, undergoes a marked thickening. A change in color under dark-field illumination is also associated with the fertilization reaction [7]; it has not been previously determined what relationship this color change bears to the other observed changes. According to Rothschild [10] the visible cortical changes may reflect a slow component of the process that prevents polyspermy.

Moser [5] reported that electrical current could substitute for a spermatozoon in initiating the cortical reaction in *Arbacia* eggs. Since then, methods have been devised to distinguish those agents which initiate a true fertilization impulse from those which act directly upon the cortical granules and do not initiate a propagated impulse [1, 11]. The purpose of the present study was to determine which type of activating effect was caused by electrical currents on the cortical reaction in the egg of the sea urchin.

MATERIAL AND METHODS

Mature gametes of the sea urchin *Psammechinus miliaris* from the Swedish west coast were obtained by shedding from washed, excised gonads.

The surface of an ordinary glass slide was divided into compartments by viscous

[1] Post-Doctoral Fellow of the National Cancer Institute, U.S. Public Health Service, Bethesda, Maryland.

[2] Present address: Department of Zoology, University of Michigan, Ann Arbor, Michigan.

grease applied with a hypodermic syringe. Two compartments containing sea water were separated from one another by a long trough containing Shillaber immersion oil. A glass capillary of the proper length and diameter was selected so that when it contained an elongated egg, it could be placed along the oil trough with both of its ends in sea water (Fig. 1). The water level in the two sea water compartments was adjusted by means of a dropper and a strip of filter paper so that the egg remained stationary in the capillary near the middle of the oil trough. Blocks of agar were placed in each pool of sea water, and the water levels were again adjusted. Ag–AgCl electrodes were placed in the agar blocks to insure good electrical contacts. An oil immersion objective (Leitz: 22 ×) was used for observations.

Rectangular pulses of current were applied to the egg by successive closing and opening of a circuit containing a source of E.M.F.

Alternating current was supplied by a conventional RC oscillator. In all experiments the frequency was 100 cy/s.

RESULTS

Alternating current.—Current densities of $4 \times 10^{-7} - 1 \times 10^{-6}$ A passed through the capillary containing an egg brought about a vigorous cortical reaction at both ends of the elongated cell. If the current was interrupted, no impulse was propagated away from the activated ends, and the cortical granules adjacent to the walls of the capillary remained intact.

Cautious application of weak alternating current showed that a very feeble membrane elevation could be elicited. An extremely delicate vitelline

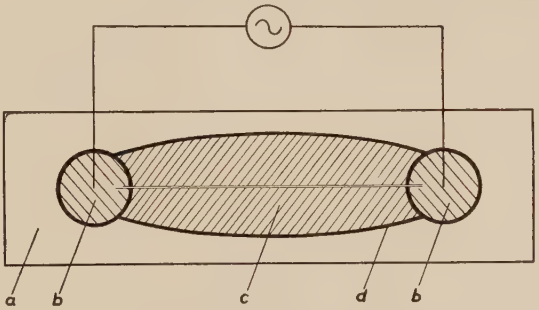


Fig. 1. A diagram of the method used in stimulating single eggs elongated in capillaries with electric current. *a*, ordinary slide; *b*, pools of sea water; *c*, trough of oil; *d*, position of the egg in the capillary; *e*, immersion oil; *f*, viscous grease.

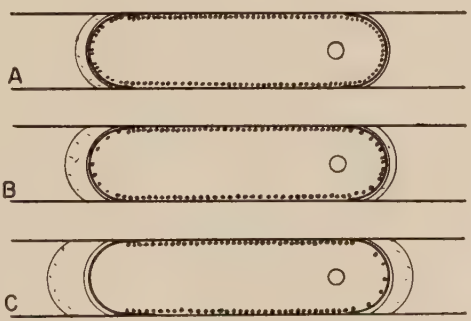


Fig. 2. A diagram to show the gradual nature of the electrically induced cortical response in an egg taken into a capillary in such a way that its animal and vegetal pole regions are at opposite ends of the cell. *A*, *B*, and *C* are progressive steps in the cortical reaction.

membrane without measurable birefringence arose only a few microns from the egg surface. If the current was not increased, the process stopped. With a high-power oil immersion (Zeiss: 60 ×), it could be seen that a few of the cortical granules had exploded on the surface beneath this delicate membrane (Fig. 2). An increase in the current density caused a continuation of the membrane elevation process, again accompanied by the breakdown of more cortical granules. The cortical response could thus be started and stopped voluntarily until the fertilization membranes on the two ends had arisen to their maximum height. At no time was there an indication that the cortical response had spread to the surface adjacent to the capillary walls, except during cytolysis caused by prolonged stimulation. The first one or two times that the cortical reaction was initiated or resumed, the egg surface exhibited the same type of contraction often seen during the normal cortical reaction to sperm attachment.

Parallel observations were carried out in dark-field illumination. When an incipient response had taken place and had been interrupted by breaking the current, it could be perceived afterward that there had been a loss of some of the orange-yellow color normally present in the unfertilized egg. Progressive steps in the elevation of a fully-extended fertilization membrane were correlated with progressive loss of the orange-yellow color and attainment of a silvery-white surface. This color change corresponded completely with that observed following fertilization [7].

There was a marked correlation between the number of cortical granules broken down and the volume of the vitelline space. It was clear also that the fertilization membrane became thicker and more refractile as it increased in height. Observations with the polarization microscope showed a clear relationship between the height of the membrane and its birefringence. The maximum birefringence attained was the same as that expected of eggs fertilized under optimum conditions.

In some eggs, the two ends responded at the same current densities, i.e. they exhibited the same threshold of stimulation. However, in the majority of eggs, there was an appreciable difference between the thresholds on the two ends. It was soon realized that the position of the nucleus played a role in this respect, for, if the nucleus was eccentrically located, the end farthest away from it showed a lower threshold. There was even some correlation between the degree of eccentricity and the difference in the thresholds; in extreme cases, the current density required to stimulate the side adjacent to the nucleus was as much as twice that required to stimulate the other end. In one extreme case in particular, the polar bodies marked one end as the

animal pole region, and the nucleus was still very close to the animal pole surface. This egg had the most marked threshold difference of any of the fifteen studied. Fourteen of these fifteen eggs studied showed a higher threshold on the side near the nucleus; the fifteenth observation was not certain. The eggs which did not exhibit any conspicuous threshold differences between their two ends were found with their nuclei almost equidistant from the two ends.

Some eggs were allowed to stratify over isotonic sucrose under a centrifugal force of about 2000 × g. for 10 minutes, and then were drawn into capillaries in such a way that the nuclei were as close as possible to one end of the eggs. Four such eggs were tested with alternating current to determine whether the side adjacent to the nucleus would again show a higher threshold. The opposite result was obtained, i.e. the side adjacent to the nucleus this time showed a lower threshold. Thus it seems unlikely that the nucleus itself determines this threshold difference. It is more likely caused by some other polarity of the egg, such as the animal-vegetal gradient.

Observations on spherical eggs with a 50 × water immersion objective with the working aperture decreased with an objective diaphragm stop showed under dark-field illumination, that there was a slight color difference between the animal and vegetal regions when viewed with subdued light. The vegetal pole region tended to be more like the fertilized surface, or whiter and brighter than the animal pole region. These observations were confirmed on eggs inside capillaries. The difference was not striking, and at high light intensities and working apertures, it was not noticeable.

Rectified alternating current.—Rectified alternating current (100 cy/s) had substantially the same influence on the surface as alternating current. Graded responses could be obtained in the same manner as with alternating current. It was noticed that membrane elevation occurred at the anode at lower current densities.

Direct current.—Direct current caused two specific kinds of cytolysis on the anodal and cathodal sides of the egg. Anodal cytolysis described already by McClendon [4] occurred at lower current densities and was characterized by a kind of vacuolization or partitioning of the cytoplasm. Cathodal cytolysis sometimes involved incipient membrane elevation, a surface contraction (similar to that observed at fertilization and upon stimulation by alternating current), and later a general break-up of the cell before the cortical reaction was complete. Direct current applied for short pulses had a much less injurious effect on the eggs and cautious application of short pulses sometimes elicited semi-normal cortical reactions.

The capillary technique has been of advantage in the examination of the effect of current of the sea urchin egg. An attempt was also made by the use of this technique to examine possible changes in membrane potential and resistance on fertilization. A short pulse of direct current (constant voltage source) of about one tenth the strength needed to cause cytolysis of the egg was passed through the capillary. The strength of current was found by measuring the voltage drop over a calibrated resistor in the circuit. This was repeated after removal of the egg with the capillary containing only sea water. The difference in current flow allowed the calculation of resistance of the egg. In a typical experiment this was 870,000 Ω with a capillary diameter of 57μ (diameter of spherical egg 98μ). In experiments carried out later [3] it has however been shown that the membrane resistance is 1350 Ω per cm^2. The surface of the egg not in contact with the capillary wall in the case mentioned above was $1.01 \times 10^4 \mu^2$ and for the hypothetical case that the current passed only through the egg and not between the egg and the capillary wall the expected resistance should be about 2.7×10^7 Ω. Hence the resistance of the layer of sea water between the capillary wall and the egg was much lower than that of the egg itself and only about one-thirtieth of the current can have passed through the egg. The resistance of the capillary with the egg was also measured during fertilization after introduction of sperm into one end of the capillary. In some experiments the total resistance was found to increase by $^1/_3$ starting 10 to 20 seconds after the first signs of fertilization were noticeable, and this increase was observed also when partial fertilization resulted. This finding cannot be explained in any other way than by increased resistance of the layer between the egg and the capillary wall. In other cases and more often fertilization caused a decrease in resistance of the capillary with the egg, and there is no reason to assume other than that this decrease was also caused by a change in thickness of the layer between the egg and the capillary wall. Consequently the method cannot be used for measurements of relative changes in membrane resistance during fertilization.

In other experiments the potential between the ends of the capillary was measured during fertilization. If fertilization caused a change of membrane potential the result would be a temporary or in case of half fertilization possibly a more permanent difference in membrane potential between the fertilized and unfertilized part of the egg. The resulting current flow would give a potential difference in the layer of sea water between the capillary wall and the egg, which might be measured between the two ends of the capillary in contact with Ag–AgCl electrodes through sea water in agar. For recording a high gain stable DC-amplifier with cathode follower input was used. The drift of potential with the preparation connected was less than 100 μV in 10 minutes. Four successful experiments were made and in no case was any shift of potential observed on fertilization. However, these experiments do not exclude the possibility of a change of membrane potential on fertilization.

DISCUSSION

It is clear that electrical current belongs to the class of activating agents that does not initiate a true, propagating fertilization impulse, but rather bypasses this step of the cortical reaction and acts more directly upon the breakdown

of the cortical granules. For this reason it is noteworthy that other subsequent changes also take place, i.e., the dark-field color change, the increase in membrane birefringence, and the limited growth of the hyaline layer. It appears very likely that these changes are not specifically dependent upon passage of the fertilization impulse, but rather upon breakdown of the cortical granules.

The suggestion that the individual cortical granules might have a random assortment of thresholds [1] was confirmed by the stepwise nature of the response when the current density was increased in steps. The cortical granules with the lowest thresholds broke down first, and others with higher thresholds followed in order until the process was complete. It could be seen clearly under high power oil immersion objectives that there was a conspicuous size inequality among the cortical granules; perhaps this either caused or contributed to the difference in threshold.

The present experiments provide some information on the reactions taking place in the vitelline space during the first minute after fertilization, or in this case, artificial activation. It has been shown that the volume of the vitelline space varies with the number of cortical granules exploded from the cortex during activation. It thus appears likely that membrane elevation is promoted by the activity of some substance released with [cf. 2] or from the cortical granules at the time of activation. It is known that some part of the cortical granules participates in the formation of the fertilization membrane [2, 8]. Failure of the cortical material to merge with the membrane results in membranes of low birefringence. This same conclusion has been strengthened by the observation of stepwise attainment of birefringence correlated with stepwise breakdown of the cortical granules.

The finding that different parts of the surface of the egg show different thresholds for electrical current is noteworthy and raises the question if also in other aspects the surface properties may be unequal in different parts of the egg. The possibility must be considered that such a property as sperm receptivity may show an unequal distribution over the surface of the egg, cf. also [9].

SUMMARY

Eggs contained in glass capillaries were placed on a slide so that the two capillary ends were bathed in pools of sea water and the middle section of the capillary in immersion oil. Alternating currents of 100 cycles per second and current density 4×10^7 to 1×10^6 A were passed from an oscillator through the capillaries. These currents initiated a normal-appearing cortical reaction

which was confined to the ends of the eggs stimulated, showing that the effect of electric current was a direct one upon the individual cortical granules rather than through initiation of the fertilization impulse. Each cortical granule exhibited a specific threshold.

The electrically induced cortical reaction could be stopped or started at will. The various aspects of the cortical reaction could be graced: the dark-field color change, the increase in volume of the vitelline space, the increase in volume of the vitelline space, the increase in birefringence of the fertilization membrane, the thickness of the hyaline layer and the number of cortical granules present in the cortex.

Different parts of the surface of the eggs studied showed different threshold for electric current. The differences in threshold apparently were related to the animal-vegetal axis of the eggs. The vegetal hemisphere exhibited a lower threshold.

An attempt was also made to examine on single eggs in glass capillaries possible changes in membrane resistance and potential on fertilization, but the capillary technique proved inadequate for these problems.

We are indebted to Dr. Gunnar Gustafson, the head of the Kristineberg Zoological Station, for his generous provision of working facilities and sea urchin material.

REFERENCES

1. Allen, R. D., *Exptl. Cell Research* **6**, 412 (1954).
2. Endo, Y., *ibid.* **3**, 406 (1953).
3. Lundberg, A., *ibid.* in press (1955).
4. McClendon, J. E., *Am. J. Physiol.* **27**, 240 (1910).
5. Moser, F., *J. Exptl. Zool.* **80**, 447 (1939).
6. Motomura, I., *Science Repts. Tôhoku Imp. Univ. 4th Ser.* **16**, 345 (1941).
7. Runnström, J., *Acta. Zool.* **4**, 285 (1923).
8. —— *Advances in Enzymol.* **9**, 241 (1949).
9. Runnström, J. and Kriszat, G., *Exptl. Cell Research* **3**, 419 (1953).
10. Rothschild, Lord, *J. Exptl. Biol.* **30**, 57 (1953).
11. Sugiyama, M., *Biol. Bull.* **104**, 216 (1953).

BRIEF NOTES

THE EFFECTS OF FREEZE-DRYING ON THE MOLECULAR ORGANISATION IN NERVE MYELIN

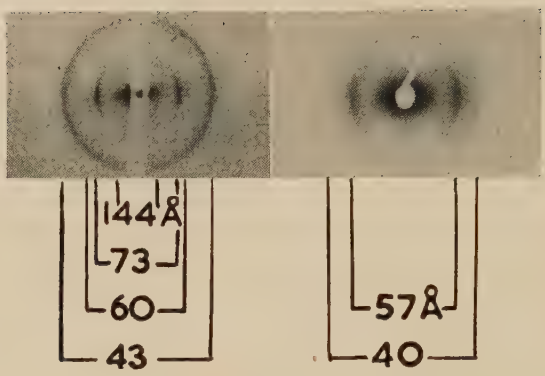
J. B. FINEAN

Department of Pharmacology, University of Birmingham, England

Received June 11, 1955

The effectiveness of freeze-drying in preserving tissues in a relatively unshrunken condition has been well demonstrated both in histological work and in electron microscopy. However, little direct information is yet available on the preservation of complex associations at the molecular level. In a previous publication [1] on the effects of drying on peripheral nerve myelin, X-ray diffraction studies indicated that the freeze-drying technique then used was no more effective in preserving lipid-protein association than was normal drying. Further recent experiments with improved freeze-drying techniques and again studying the specimens by X-ray diffraction methods have revealed a number of interesting characteristics of the effects of freeze-drying on the layered lipoprotein structure of nerve myelin. Fig. 1 compares the normal dried nerve pattern with that of a specimen freeze-dried by plunging the nerve (frog sciatic) into isopentane cooled in liquid air and then in a Gersh-type freeze-dryer at -30°C[1]. The diffraction bands in the normal dried nerve pattern have previously been accounted for [1, 2] as representing a residual lipoprotein component (73 Å band) and three separate phases of lipid, two of which (60 Å and 40 Å bands) are different polymorphic forms of the same material and are interconvertible by changes in temperature. In the freeze-dried specimen there is a strong band at 57–60 Å and a weaker one at about 40 Å. On heating, the intensity of the latter increases whilst that of the 57–60 Å band decreases, thus indicating that at least part of the material responsible for the 57–60 Å band is lipid. However, at temperatures above 150°C when the 40 Å band has become faint and diffuse (lipid liquified) a diffraction still persists at about 60 Å indicating the presence of a component which is probably not simply lipid and which may be a lipoprotein. In addition to these diffraction bands there is also a low angle particle scatter the characteristics of which can be accounted for in terms of layers of plate-like particles with a uniform thickness of 130–140 Å. This would appear to replace the customary residual lipoprotein spacing in the normal dried nerve pattern, and in fact such a diffraction band can be obtained by re-wetting the freeze-dried specimen and drying in the normal way. It would seem likely that the low angle scatter comes from lipoprotein layers which have been prevented from collapsing onto each other when the water was removed, and thus form a discontinuous system which cannot produce diffraction. In this way overall shrinkage of the nerve is reduced by freeze-drying but

[1] I am grateful to Dr. K. Aterman of Dept. of Anatomy for freeze-drying these specimens.



the diffraction patterns show that part of the lipid still separates from the lipoprotein system when the specimen is dried. Slower freezing or drying at a higher temperature leads to an increase in intensity of the 40 Å band relative to that of the 60 Å band.

REFERENCES

1. Elkes, J. and Finean, J. B., *Discussions of the Faraday Society*, No. 6 (Lipoproteins), p. 134 (1949).
2. Finean, J. B., *Exptl. Cell Research* **5**, 202 (1953).

NUCLEAR CHANGES IN THE GROWTH CYCLE OF *CARYOPHANON LATUM*

A. A. TUFFERY[1]

Department of Bacteriology, University of Birmingham, England

Received December 14, 1954

The giant bacterium *Caryophanon latum* was first described as a coencytic filament by Peshkoff [8], and more fully investigated by Pringsheim and Robinow [9] who showed that it consisted of numerous disc-shaped cells, separated by cross-walls, and each containing a nucleus in the form of a pair of longitudinally dividing rods, closely resembling the now classical type of bacterial nucleus. In large cells found in older cultures the nuclei appeared as rings or discs. The nuclear development cycle of this interesting microorganism has not, however, so far been described, and it is the purpose of this paper to do so. A record of the process in *Caryophanon* is of some importance because its large size (up to 3μ in diameter) enables the details to be observed with much greater clarity than in the majority of bacteria.

[1] Present address: Microbiological Research Department, Porton, Wiltshire, England.

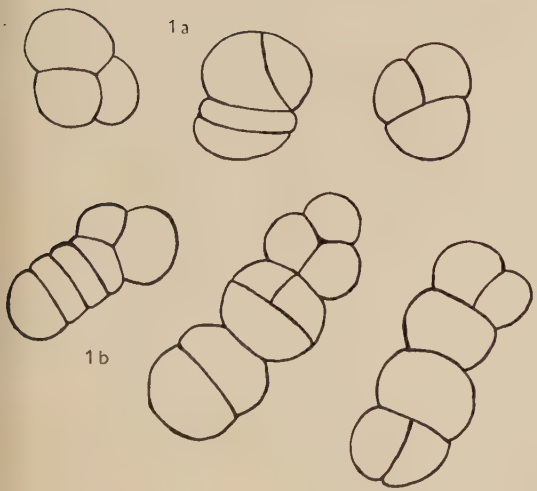


Fig. 1. Resting stages. Hale's method.

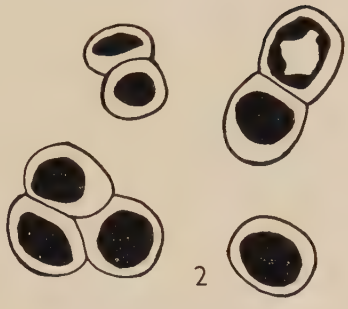


Fig. 2. Resting stages, stained to demonstrate nuclear structures. Acid-Giemsa.

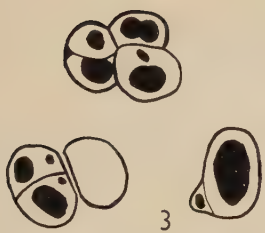


Fig. 3. Germinating resting cells, showing polar nuclei. Acid-Giemsa.

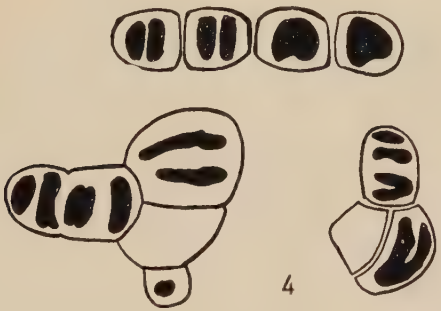


Fig. 4. Germinating resting cells, slightly older than those in Fig. 3, and showing young vegetative bacilli forming. Acid-Giemsa.

Materials and methods.—A strain of *Caryophanon latum* was isolated from cow-dung by the methods of Pringsheim and Robinow [9], and was cultivated at 28°C aerobically upon a medium consisting of 0.5 per cent '*Difco*' yeast extract, 0.5 per cent '*Difco*' peptone, and 0.01 per cent sodium acetate, with 2 per cent agar.

Inocula were taken from three-day-old cultures, consisting largely of resting stages, and their growth in sub-culture was examined at various stages from 1 to 48 hours.

Smears made upon cover slips were permitted to dry for a few minutes at room temperature and were stained either by Hale's [4] technique for cell walls or by Robinow's modification of the Feulgen reaction [9]; hydrolysis for 10 minutes at 60°C in *N*/1 HCl was adequate for all stages except resting cells, which sometimes required as much as 25 minutes. Staining was also conducted at 60°C for 4 mins. in Giemsa solution (Gurr's R. 66, at 1:10).

Fig. 5. Trichomes showing narrow, disc-like cells. Hale's method.

Fig. 6. Trichomes, showing typical rod-shaped nuclei, dividing longitudinally. Acid-Giemsa.

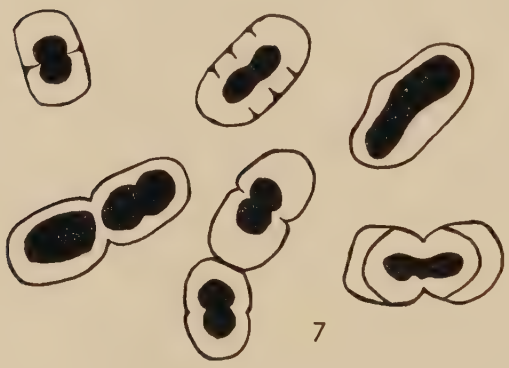


Fig. 7. Cells from old cultures, showing fusion of nuclei. Acid-Giemsa.

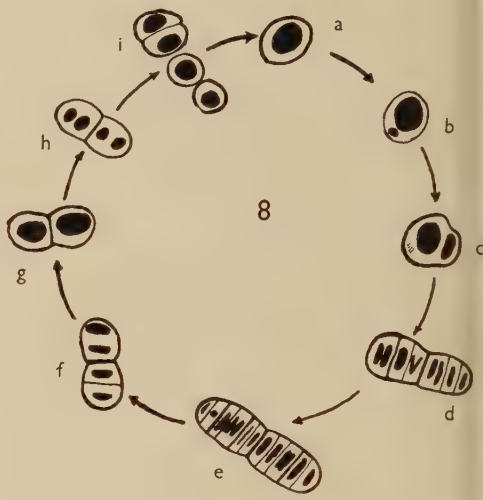


Fig. 8. Diagram illustrating the life-cycle of *Caryophanon*.

Because of their thickness and irregular multicellularity, satisfactory photomicrographs of these structures could not be made.

Observations.—The resting stages, of which mature cultures mainly consisted, were approximately spherical, about 3μ in diameter, but could readily be shown by Hale's cell wall stain to consist of groups of from 1 to 4 cells; these cells were very irregular, both in size and in arrangement (Fig. 1, A). Their nuclear structures were spherical or irregularly polygonal, vesicular or reticulate and, at first, very difficult to stain. When sub-cultured upon fresh medium the first sign of germination, after about 2 hours incubation, was an increased affinity for the stain (Fig. 2).

When the nucleus commenced to grow and divide, the initial division consisted in the ejection of a small daughter nucleus, apparently analogous to a polar body, which subsequently degenerated (Fig. 3). This closely resembled the nuclear reduction process observable in other bacteria [2, 3, 5, 6, 7]. Thereafter the nucleus divided to form the characteristic transverse rods of the bacterial vegetative phase [10],

which are already well-known to occur in *Caryophanon* [8, 9]. Each cell of the resting stage initiated a trichome, so that growth was, at first, very irregular (Fig. 4), with bacillary forms arising at various angles to each other.

From 6 to 36 hours, approximately, the cultures consisted mainly of vegetative bacilli, each in the form of a trichome of narrow, disc-like cells, separated by cross-walls, clearly visible by Hale's method (Fig. 5), and containing paired, rod-like nuclei, dividing reductionally in typically bacterial fashion (Fig. 6), and closely resembling those which can so clearly be demonstrated in the very similar, but even larger *Oscillospira guilliermondii* [11, 12].

Between 36 and 38 hrs. incubation the number of resting stages in the culture progressively increased. During this process, the nuclear material in some trichomes appeared to undergo a process of fusion centrally (Fig. 7), in a manner reminiscent of the early stages of resting cell formation in other bacteria [1, 3]. The trichomes then segmented and the segments became rounded off to form resting stages (Fig. 1, B) thus completing the cycle. The entire cycle is shown diagrammatically in Fig. 8.

Summary and Conclusions.—The giant bacterium, *Caryophanon latum* is shown to undergo a cycle of development with nuclear changes which confirms the general scheme of such cycles in bacteria which has been proposed independently by several authorities [1, 2, 3, 5, 6, 7].

The resting nucleus is apparently globular and vesicular; the vegetative nucleus is in the form of reductionally dividing rodlets, and the cycle includes processes strongly suggestive of nuclear fusion, and of nuclear reduction by the ejection of a small daughter nucleus, resembling a polar body. The latter process, however, appears to be exceptional in occurring in the course of the germination rather than the maturation of the resting nucleus.

These observations serve to confirm not only the main features of the bacterial nuclear cycle, but also the systematic position of the genus *Caryophanon*. Like *Oscillospira* [11, 12] which it so closely resembles, it is entirely bacterial in its main characters.

REFERENCES

1. Bisset, K. A., *J. Hyg.* **47**, 182 (1949).
2. Bisset, K. A., Grace, J. B., and Morris, E. O., *Exptl. Cell Research* **2**, 388 (1951).
3. Grace, J. B., *J. Gen. Microbiol.* **5**, 519 (1951).
4. Hale, C. M. F., *Lab. Practice* **2**, 115 (1953).
5. Klieneberger-Nobel, E., *J. Hyg.* **44**, 99 (1945).
6. —— *J. Gen. Microbiol.* **1**, 33 (1947).
7. Morris, E. O., *J. Hyg.* **49**, 46 (1951).
8. Peshkoff, M. A., *J. Gen. Biol.* **1**, 598 (1940).
9. Pringsheim, E. G. and Robinow, C. F., *J. Gen. Microbiol.* **1**, 267 (1947).
10. Robinow, C. F., Addendum to The Bacterial Cell by R. J. Dubos. Harvard University Press (1945).
11. Tuffery, A. A., *J. Gen. Microbiol.* **10**, 342 (1954).
12. —— *Nature, London* **174**, 838 (1954).

OBSERVATIONS ON THE ULTRASTRUCTURE OF EXPERIMENTALLY ALTERED NERVE FIBRES, GROWN AND INJURED IN TISSUE CULTURES[1]

H. MEYER

Instituto de Biofísica da Universidade do Brasil, Rio de Janeiro, Brasil

Received May 25, 1955

In a previous paper the results of an electron microscopic study of nerve fibres, grown in tissue cultures, have been discussed [2]. It has been shown that the cytoplasm of these fibres has a fine longitudinal striation which can be followed up to the growing tips of the fibres. Mitochondria-like inclusions and vacuol-like holes can be found among the striations.

We know that cultures of nervous tissue must be treated with special care. Slight injuries or damages during manipulation of the cultures have an immediate effect upon the aspect of the fibres [1]. Varicosities are formed and swellings which may be reversible, but which in case of severe damage may lead to a rapid degeneration of the distal part of the fibres.

Swellings or fusiform enlargements in the nerve fibres may also occur normally in the tissue cultures, and we saw that those which are thin enough for the penetration of the electron beam have the same longitudinal striation which the fibre has. There are also others which show no structure at all, and in more recent observations inclusions have been found in them in form of droplets, or mitochondria-like filaments. These formations do not always represent pathological alterations of the nerve fibres, since they are found in fibres which are in perfect condition with regard to structure and growth.

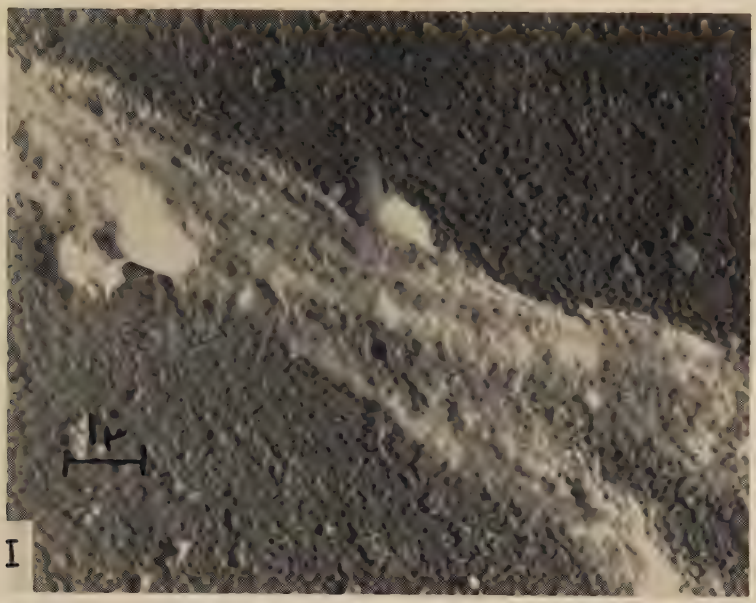
For a better understanding as a whole, and to avoid future errors, we thought it interesting to injure some cultures and examine in the electron microscope the effect of the damage upon the structure of the nerve fibres, then compare the aspect of the experimentally altered fibres with that of the intact ones.

Material and Technique.—In preparing the tissue cultures the same material and technique were used as in the earlier work for the normal fibres [2]. Spinal ganglia of 9 to 14 days old chick embryos were cultivated in a thin plasma clot on covers slips which had been coated previously with Formvar. The incubation temperature was 33 35°. When the cultures showed a good growth of fibres, a slight cut was made in

[1] This investigation has been carried out in the *Instituto de Biofísica da Universidade do Brasil*, Rio de Janeiro (Dir. Dr. Carlos Chagas) under the tenure of a *Conselho Nacional de Pesquisas* fellowship.

Fig. 1. Spinal gangl. of 14 days old chick embryo. 7 minutes after an injury. Striation in cytoplasm still preserved, droplets appear along the fibres and are extruded. ×10,000.

Fig. 2. Spinal gangl. of 14 days old chick embryo. Terminal extension of intact fibre (*a*) with membranes and fine prolongations. In (*b*) terminal extension one hour after an injury. ×1500 and 3000.


I

2a
b

the centre of the explant, seat of the nerve cells, with the point of a new cataract knife. Care was taken not to push the plasma clot too much.

The effect of this injury could be observed immediately in the peripheral part of the cultures, where the nerve fibres showed the alterations which are described above. The cultures were fixed from two to 10 minutes after the injury, some after one hour, in vapour of osmic acid, with the same precautions and care which had been observed during the fixation of the normal fibres [2]. After washing in dist. water, and after removing the plasma clot the formvar film with the remaining fibres was transferred to the electron microscope grids, according to the technique of Porter, Claude and Fullam [3]. Here they were shadowed with chromium. They were examined then in the "Philips" electron microscope with 60 and 80 kV.

Observations.—When the injury in the centre of the culture was restricted to a small area not all the nerve cells or fibres were damaged, and not all of the fibres in the periphery react. In those which are altered the aspect varies from complete destruction to more or less pronounced alterations. In all cases we see droplets which seem to be extruded from the cytoplasm, appear along the fibres (Fig. 1), often at quite regular distances. They tend to flatten with time and stain with osmic acid. In some cases the fibrillar aspect of the cytoplasm becomes more pronounced by this loss of material, in others the structure is completely lost. Also in the terminal extensions of the fibres the effect is immediate. The ameboid membranes retract and enlargements appear along the thin prolongations (Fig. 2*a* and *b*).

In the fusiform enlargements of the fibres which occur normally in tissue cultures the striation may be partially preserved after an injury. The enlargements may also show extrusion of droplets, or the structure may be completely lost.

Results and Discussion.—The effect of damage on the tissue cultures is immediate and varies in degree on the fibres, on their terminal extensions, and on the enlargements which preexisted in the fibres. The most immediate and striking effect is the apperance and extrusion of droplets along the fibres. They are probably of lipid nature because they stain black with osmic acid. Judging from their rather regular distribution the droplets might be responsible for the varicosic aspect of the fibres in the light microscope immediately after an injury.

The results obtained during this investigtion are only preliminary. For technical reasons we thought it necessary to examine in a rough test the alterations in the structures of the injured fibres, because similar damages are easily produced by inadequate treatment of the tissue cultures, and similar changes in the structures will also occur during the preparation of peripheral nerves or of the central nervous system for inclusion or ultrathin sectioning, since it is almost impossible to obtain such preparations without an interruption of the nerve fibres' continuity.

REFERENCES

1. Levi G. and Meyer, H., *J. Exptl. Zool.* **99**, 141 (1945).
2. Meyer, H., *Exptl. Cell Research* **7**, 15 (1954).
3. Porter, K. R., Claude, A., and Fullam, E. A., *J. Exptl. Med.* **81**, 233 (1945).

ACTIVITÉ MITOTIQUE PROVOQUÉE PAR LA THIOURÉE ET TENEUR EN ACIDE DÉSOXYRIBONUCLÉIQUE DE LA CELLULE HÉPATIQUE

J. FAUTREZ, E. PISI et G. CAVALLI

Laboratoire d'Anatomie humaine de l'Université de Gand, Belgique, et Institut de Clinique médicale générale et de Thérapeutique médicale de l'Université de Bologne, Italie

Reçu le 31 mars, 1955

Après Lison et Pasteels [3] les auteurs de ces pages [1] ont eu l'occasion de montrer que la teneur en DNA des noyaux intercinétiques de certains tissus animaux augmente notablement à l'occasion d'un accroissement important de l'activité mitotique. Ce phénomène est cependant loin d'être généralement admis; nombreux sont au contraire les auteurs qui sont d'avis que la teneur en DNA des divers noyaux intercinétiques est rigoureusement constante, indépendante de l'activité tant mototique qu'anabolique. C'est dans cet esprit que Swift [5] suggère une cause d'erreurs possible : les teneurs moyennes en DNA plus élevées, que l'on mesure à l'occasion d'une activité mitotique accrue, seraient simplement dues à la présence de nombreux éléments préprophasiques, qui auraient déjà commencé leur synthèse de DNA en rapport avec la mitose. Que cette cause d'erreurs, qui pourrait fortement déplacer la moyenne vers le haut, n'intervient pas nécessairement, peut être démontrée par les résultats qui nous voulons communiquer ici.

Rachmilewitz, Rosin et Doljanski [4] ont attiré l'attention sur la possibilité de produire dans le foie du Rat sous l'effet de la thiourée une onde mitotique nettement délimitée dans le temps. Dans des conditions expérimentales légèrement modifiées il nous a été possible de confirmer cette observation.

A 6 rats blancs fut administrée une dose journalière de 10 mg/100 g de thiourée. Deux d'entre eux furent tués à 1½ jour, deux autres à 2 jours, et les deux dernières à 2½ jours après la première injection. Tous les rats, ainsi que les 2 témoins reçurent une dose unique de colchicine (0,2 mg/100 g) 9 heures avant la mort. Comme l'indique, Tableau I, il est possible de saisir dans ces conditions une onde mitotique à maximum vers la fin du 2e jour et pratiquement terminée après 2½ jours.

Nous nous sommes dès lors demandé si cette onde d'activité mitotique intense, mais nettement délimitée dans le temps, influencerait la teneur en DNA des noyaux hépatiques intercinétiques, et si notamment cette teneur serait plus élevée après cette onde que dans des foies témoins.

A 5 rats (R 229–233) fut administrée une dose journalière de 10 mg/100 g de thiourée. Les animaux furent tués 3 jours après le début de l'expérience. Comme les 5 témoins (R 224–228) ils reçurent 9 heures avant la mort une dose unique de colchicine (0,2 mg/100 g). Les foies des divers animaux ainsi que le testicule du témoin R 224 furent fixés à l'alcool-formol-acétique, déshydratés, inclus simultanément. Des coupes de ces divers organes furent portés sur une même lame porte-objet afin de s'assurer une réaction de Feulgen absolument homogène pour tous. Notons que dans les divers foies l'index mitotique reste inférieure à 1. Nous avons déterminé la teneur

TABLEAU I

Index mitotique du foie après traitement à la thiourée.

Cet index fut obtenu en dénombrant toutes les mitoses contenues dans une coupe de 10 μ d'épaisseur et en calculant, après mesure de la surface de la coupe le nombre de mitoses par surface de 10 mm².

N° de l'animal	243	242	241	240	239	238	237	236
Durée de l'expérience	témoins		1 ½ jour		2 jours		2 ½ jours	
Index mitotique	0,8	0,7	3,2	2,7	8,0	12,4	1,3	1,0

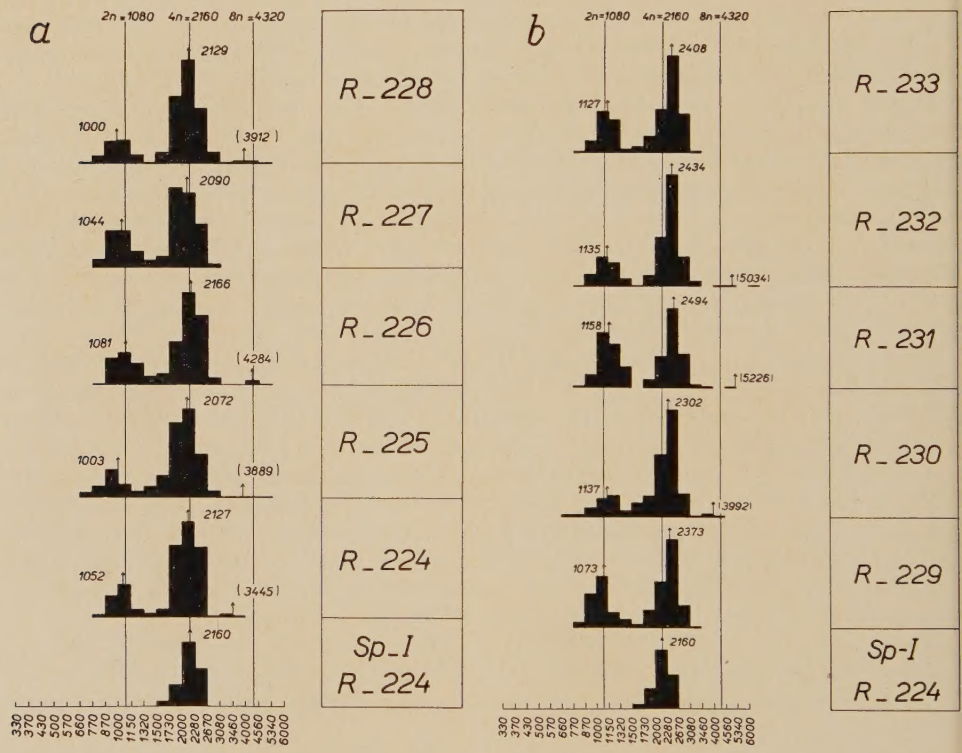


Fig. 1. Histogramme de la teneur en DNA des noyaux intercinétiques de foie de rats témoins et de rats traités à la thiourée.

en DNA des noyaux intercinétiques par la méthode histophotométrique de Lison (pour les détails de la technique, cf. [2]). Comme cette méthode ne donne qu'une teneur relative en DNA nous avons déterminé comme valeur de reférence la teneur moyenne de 100 jeunes spermatides de 1er ordre qui contiennent 4 fois autant de

TABLEAU II

Teneur moyenne en DNA des noyaux intercinétiques des foies de rats témoins et de rats traités à la thiourée.

		Classe $2n$			Classe $4n$		
		N	$x \pm s\bar{x}$	s	N	$x \pm s\bar{x}$	s
Foies des témoins	R 224	54	1052,34 ± 16,7	122,7	193	2127,45 ± 15,9	221,8
	R 225	57	1003,20 ± 24,7	186,5	188	2071,70 ± 20,5	281,1
	R 226	68	1081,12 ± 16,07	132,5	177	2166,39 ± 20,2	269,4
	R 227	83	1044,36 ± 16,1	147,4	167	2089,51 ± 20,0	258,5
	R 228	51	1000,24 ± 18,9	135,02	192	2129,17 ± 18,9	263,4
	Pool	313	1038,25 ± 8,5	150,5	918	2116,04 ± 8,6	262,8
Foies des animaux traités á la thiourée	R 229	100	1073,40 ± 15,4	154,08	150	2372,60 ± 23,7	890,7
	R 230	53	1136,75 ± 26,05	189,6	193	2302,10 ± 22,7	315,5
	R 231	114	1158,00 ± 12,50	133,8	135	2494,41 ± 26,1	304,3
	R 232	67	1134,81 ± 16,8	137,8	180	2433,67 ± 21,6	289,9
	R 233	80	1127,00 ± 15,06	134,7	170	2408,47 ± 23,01	200,0
	Pool	414	1125,10 ± 7,4	150,5	828	2395,80 ± 10,7	308,2

DNA que le spermatozoïde ou que la valeur haploïde théorique (n). C'est à partir de ce résultat que furent déterminées les valeurs $2n$, $4n$, $8n$ théoriques. Les histogrammes de la figure suivante résument nos résultats. On y trouve en abcisses la teneur en DNA (en unités arbitraires rangées selon une échelle logarithmique) et en ordonnées le nombre de noyaux contenant une quantité de DNA comprise entre 2 chiffres de l'échelle horizontale. Pour chaque foie 250 mesures furent effectuées.

On reconnait les 3 classes de noyaux du foie de rat ($2n$, $4n$, $8n$) : cette dernière est quantitativement trop peu importante pour permettre une analyse statistique et sera négligée plus loin. Pour les noyaux des deux autres classes, on constate que la moyenne statistique de leur teneur en DNA se rapproche chez les 5 témoins (A) fortement des valeurs $2n$ ou $4n$ théoriques. Chez les 5 animaux traités à la thiourée (B) cette moyenne est nettement plus élévée que chez les témoins. L'analyse statistique montre que ces résultats correspondent à une augmentation en DNA hautement significative. En effet Tableau II résume les données de cette analyse.

En appliquant entre les pools des résultats pour les foies témoins et pour les foies expérimentés le test t de Student on obtient pour les noyaux de la classe $2n$ une valeur de $t = 7{,}75$ et pour ceux de la classe $4n$ une valeur de $t = 20{,}4$. Ceci correspond dans les deux cas à une valeur de $P < 0{,}001$, donc à une différence hautement significative.

Ces résultats montrent clairement que l'onde mitotique provoquée dans le foie de Rat par la thiourée est accompagnée d'une augmentation de la teneur en DNA des noyaux intercinétiques. Les conditions dans lesquelles les mesures sont effectuées

(immédiatement après l'onde mitotique) excluent des erreurs dues à l'influence sur la moyenne de la présence de nombreux éléments préprophasiques, éventuellement plus riches en DNA.

BIBLIOGRAPHIE

1. Fautrez, J., Cavalli, G. et Pisi, E., *Nature* (sous presse).
2. Lison, L., *Acta Anat.* **10**, 333 (1950).
3. Lison, L. et Pasteels, J., *Arch. Biol.* **62**, 1 (1951).
4. Rachmilewitz, M., Rosin, A. et Doljanski, L., *Am J. Pathol.* **26**, 937 (1950).
5. Swift, H., *Phys. Zool.* **23**, 169 (1950).